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Actions of l-methyl-3-isobutyl Xanthine on the Cat Neuromuscular Junction

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ACTIONS OF 1-METHYL-3-ISOBUTYL XANTHINE ON THE CAT NEUROMUSCULAR JUNCTION

by

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BIOGRAPHY

Alan C. Roman was born on June 22, 1947 in Chicago, Illinois. In June, 1965 he graduated from St. George High School, Evanston, Illinois.

In September, 1965, he entered Loyola University, Chicago and in June, 1969, received a Bachelor of Science degree with a major in biology.

He began his graduate training in September, 1969, at Loyola University, Chicago where in June, 1971, he received a Master of Science degree in biology. He was voted a member of Tri-Beta Biological Honor Society in 1970, and was also awarded a graduate teaching assistantship from September, 1970-May, 1971.

In September of 1972, Mr. Roman was accepted as a graduate student in the Department of Pharmacology at Loyola University Medical Center. He was a student of Dr. R.S. Jacobs until June, 1974 at which time Dr. A.G. Karczmar became his principal adviser. In 1978, Mr. Roman accepted a position with Smith-Kline and French Laboratories as a Regional Medical Associate for the Great Lakes region where he is currently employed.

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$\mathring{A}$	angstrom unit
ACh	acetylcholine
AChE	acetylcholinesterase
A.P.	action potential
^{o}C	degrees centigrade
Ca	calcium
$[Ca^{++}]$	calcium ion concentration
cAMP	cyclic-3',5'-adenosine monophosphate
ChE	cholinesterase
cm	centimeter
CO_2	carbon dioxide
CoA	coenzyme A
C.V.	coefficient of variation
dm	mobilization rate
d-Tc	d-tubocurarine
EPP	endplate potential
E_K	potassium equilibrium potential
E_{Na}	sodium equilibrium potential
head	the first fifteen EPPs of a tetanus
i.v.	intravenous route of administration
K	potassium
kg	kilogram
KΩ	kilohm
m_o	quantal content of the first EPP of a tetanus

m_t	quantal content of the tail of the train of EPPs
MEPP	miniature endplate potential
mg	milligram
$[Mg^{++}]$	magnesium ion concentration
min	minute
MIX	1-methyl-3-isobutyl xanthine
ml	milliliter
mm	millimeter
msec	millisecond
mV	millivolt
MΩ	megohm
N	number of samples in a population
n	readily releasable store
$[Na_i]$	sodium ion concentration inside a cell
$[Na_o]$	sodium ion concentration outside a cell
O_2	oxygen
p	probability
pH	negative log of hydrogen ion concentration
q	quantal size
RMP	resting membrane potential
S.D.	standard deviation
sec	second
tail	the EPPs of a tetanus excluding the head
μ	micrometer (micron)
μ^2	square micrometer

μF	microfarad
μg	microgram
μsec	microsecond
Ω	ohm

CHAPTER I

INTRODUCTION

A Statement of Purpose

The primary aim of my research is to investigate the pharmacological actions of 1-methyl-3-isobutylxanthine (MIX) with regard to the sites and mechanisms of its action upon neuromuscular transmission. An undertaking such as this requires not only a knowledgeable utilization of precise and sensitive electronic apparatus, but demands, at least, a basic understanding of neuromuscular physiology. Some aspects of neuromyal transmission encompass several specialized areas of research. For example, one such area concerns biochemical and pharmacological aspects of neurotransmitter synthesis and storage. Cholinergic receptor studies, localization and function of cholinesterase or cyclic nucleotide research are yet other specialized areas directly related to junctional physiology. These areas, and other appropriate aspects of neuromuscular transmission are discussed in the first part of this Introduction. These aspects of neuromyal transmission are integrated so as to familiarize the reader with normal neuromyal transmission and to facilitate the understanding of transmission altered by MIX.

An exhaustive description of any defined aspect relating to neuromuscular transmission is nearly impossible in this Introduction. The reader is thus referred to the following reviews: Thesleff and Quastel

(1965), Katz (1966), Nastuk (1966), Karczmar (1967), Riker and Okamoto (1969), Hubbard (1970) and Zaimis (1976). These authors have compiled extensive and detailed documentation of physiological and pharmacological aspects of neuromuscular transmission.

The second part of this Introduction deals with the pharmacology of MIX and related xanthines. In this part, the effects of MIX at sites other than nerve or striated muscle are reviewed since no information has been published as to the neuromuscular actions of this drug. However, junctional effects of other xanthines are presented; the pertinent review provides additional support for proposed sites of MIX action at the neuromuscular junction.

Altogether, the purpose of this Introduction is to represent xanthines as capable of affecting specific sites and/or biochemical mechanisms at the neuromyal junction, resulting in the facilitation of transmission. The Introduction also serves to provide scientific documentation or a basis for my investigations of MIX.

Membrane Concept

Early anatomical studies have shown that cell membranes in general consist of a bimolecular layer of lipid surrounded by protein (Davson and Danielli, 1943). In excitable cells this membrane functions as an insulator interposed between two conducting media, the extracellular electrolyte and the intracellular cytoplasmic gel (Katz, 1966). Both nerve and muscle cell may be visualized as cylindrical conductors whose membranes consist of resistive (pore) components and capacitative areas (ascribed to the lipoprotein membrane). This formulation underlies the

comparison of the excitable cell to a cable conductor (Cole and Hodgkin, 1939; Katz, 1948; Falk and Fatt, 1964). Conducted signals along excitable membranes, however, become distorted due to the resistive and capacitative leakages through the membrane and because of energy dissipation in the fiber core (Katz, 1966). An awareness of cable properties is essential for the basic understanding of the membrane dependent control of conducted potentials and on the maintenance of resting membrane potentials.

In general, the resting membrane potentials (RMPs) range from -60 to -90 mV in both nerve and muscle. RMPs are largely dependent on the electrochemical forces controlling the ratios of intracellular and extracellular Na^+, K^+ and Cl^-, and on the semipermeable nature of the cell membrane. Bernstein (1902) erroneously suggested that resting cell membranes were selectively permeable to potassium ions alone, and that their efflux was opposed by extracellular Na^+. He also proposed that K^+ was attracted by the presence of intracellular anions not capable of moving across the cell membrane. Bernstein concluded that during an action potential, a simple loss of ionic selectivity occurred; this resulted in a neutralization of RMP.

Boyle and Conway (1941) subsequently observed that frog sartorius muscle cells selectively accumulated K^+ and Cl^- while excluding practically all Na^+. RMP was attributed to the balance of electrical and osmotic forces acting on Na^+, K^+ and Cl^-. Distribution of the ions followed the law of Donnan equilibrium, as the large organic anions synthesized intracellularly affected the distribution of the small K^+

and Cl^- so that at equilibrium $[K_i^+]/[K_o^+] = [Cl_o^-]/[Cl_i^-]$*.

Investigations of frog muscle fibers showed that the permeability of the membrane to Na^+ amounted, under resting conditions, to 5% of that of K^+ (Hodgkin and Horowicz, 1959). Experiments with labeled ions conclusively demonstrated that inward and outward fluxes of both Na^+ and K^+ occur in resting muscle fibers (Ussing, 1947; Hodgkin and Horowicz, 1959). Furthermore, the active Na^+ efflux was shown to be coupled to K^+ influx; this process could be inhibited by metabolic inhibitors (Hodgkin and Keynes, 1955).

Relative internal and external concentrations of ions in resting nerve or muscle were noticeably different (Webb and Young, 1940). In resting cells, extracellular Na^+ concentration is about ten times greater than its intracellular concentration. In contrast, the concentration of extracellular K^+ is fifteen to thirty times less than its intracellular concentration while external Cl^- concentration is from six to forty times greater than internal Cl^- concentration; actual ratios depend on cell type. Finally, intracellular negativity is supplied largely by anions such as organic phosphates, amino acids and polyelectrolyte proteinates (Katz, 1966).

The pioneering investigations of Bernstein (1902) led to conclusive theories of ionic distribution. On the basis of the biophysical principles of Nernst and Ostwald ion distribution could be related to potential difference in terms of the Nernst Equation:

* The i and o subscripts designate intracellular and extracellular ionic concentrations, respectively.

$$E_A = RT/F \ \ln \frac{\text{ion A out}}{\text{ion A in}} *$$

This equation expresses the value of the potential difference across the cell membrane at equilibrium (E_A) for the ionic species (A). By substitution of Na^+, K^+ or Cl^- for A, the equilibrium potentials (E_{Na^+}, E_{K^+} or E_{Cl^-}) for these ionic species can be easily calculated. In muscle cells, the RMP was found to be close to the equilibrium potential for K^+ (E_{K^+}). However, for nerve, none of the equilibrium potential determinations for Na^+, Cl^- or K^+ coincided with the value of the observed RMPs. This finding suggested that RMP of the nerve membrane was not dependent on any single ionic species.

By combining flux equations for individual ions, Goldman (1943) arrived at the axiom that at resting membrane potential there was no net movement of charge across the cell membrane. Further calculations by Hodgkin and Katz (1949) incorporated permeability factors into individual current equations for each ionic species. Altogether, the membrane potential could be described by the Goldman-Hodgkin-Katz equation:

$$V = RT/F \ \ln \frac{P_K [K_o] + P_{Na} [Na_o] + P_{Cl} [Cl_i]}{P_K [K_i] + P_{Na} [Na_i] + P_{Cl} [Cl_o]}$$

where

V = the resting membrane potential
P = permeability of the ion designated by the subscript, and i and o refer to intracellular and extracellular designations, respectively (cf also p9).

* R is the universal gas constant
T is the absolute temperature
F is "Faraday" or the electric charge per gram equivalent of univalent ions.

Thus, RMP was shown to depend on the intracellular and extracellular concentrations of Na^+, K^+ and Cl^- and their relative permeabilities.

Nerve Impulse Initiation

Evoked transmitter release at the myoneural junction is dependent upon the arrival of a nerve action potential at the motor nerve terminal. Furthermore, the ionic fluxes responsible for the nerve action potential as well as the end plate potential are similar despite other contrasting qualities of these potentials. Gradual physiological discoveries of action potential mechanisms have preceded the more contemporary investigations of neuromuscular transmission. Hence, some of the techniques used to analyze action potentials have some relevance to the synaptic potentials as well. For these reasons, a review of early nerve conduction will precede the subsequent account of neuromuscular transmission.

Application of a local subthreshold current pulse to a resting nerve results in a graded local depolarization or displacement of the RMP (Rushton, 1932; Hodgkin, 1937; Katz, 1938). As a consequence of cable properties the voltage signal fades in an exponential manner with increasing distance from the stimulus (Katz, 1937). If the local depolarization reaches a critical threshold level in the course of a rapid displacement of RMP from -80 to -50 mV, a spike or action potential ensues (Rushton, 1937; Katz, 1966). This signal is self propagated and is non-decremental in its travel along any given length of the nerve. Inactive areas of the nerve adjacent to the action potential front are activated by the spread of the action current resulting in nerve impulse propagation (Hodgkin, 1937; Rushton, 1938).

Results of early investigations of nerve impulse conduction supported the concept of ionic mechanisms being the underlying basis of the action potential. For example, Bear and Schmitt (1939) and Webb and Young (1940) demonstrated the presence of significant concentration gradients for both K^+ and Cl^- between the axoplasm of squid giant axon and either blood or sea water. Action potentials of these axons were recorded as a function of extracellular K^+ concentrations; the potential spike heights were inversely proportional to extracellular K^+ levels and action potentials were totally abolished at a K^+ concentration between 2.5 and 6 times normal (Curtis and Cole, 1942).

Axonal Na^+ and Cl^- concentrations were observed by Steinbach and Spiegelman (1943) to be one tenth of those in sea water. Subsequent experiments by Hodgkin and Katz (1949) revealed that Na^+ was essential for the integrity of the action potential and alteration of external Na^+ concentration could affect action potential amplitude as well as rate of rise and conduction velocity. However, an earlier finding was that the replacement of all ions with isosmotic dextrose did not affect the resting or action potential of squid axon (Curtis and Cole, 1942); the idea that extracellular ions were unnecessary for nerve conduction was later rejected following the discovery of an impermeable layer present in perineurium which retained the ions (Hodgkin, 1976). Furthermore, in other experiments, crab axons placed in salt free media became inexcitable (Katz, 1947) which agreed with the Na^+ hypothesis of Hodgkin and Katz (1949).

Resting nerve exhibits significant permeability to K^+ but very little permeability to Na^+. During the action potential rising phase,

somewhat the reverse of this situation occurred whereby normal selective permeability was diminished resulting in a 500 fold increase in Na^+ permeability with little initial change in K^+ or Cl^- permeability (Hodgkin and Katz, 1949). [Following Na^+ influx, the K^+ permeability increases in a gradual but prolonged manner (Hodgkin and Huxley, 1952d)]. The rapid decline of RMP to zero was at first explained by the Na^+ influx in response to its concentration gradient in the presence of increased membrane permeability. This concept of membrane potential neutralization by an action potential was first advocated by Bernstein (1902). However, several investigators revealed that the nerve was not merely neutralized but actually underwent polarity reversal (Hodgkin and Huxley, 1939, 1945; Huxley and Stampfli, 1951). This reversal, termed overshoot, contradicted Bernstein's concept of membrane neutralization during an action potential. Overshoot was later explained by the depolarization-related increase in Na^+ conductance (g_{Na}) thereby moving towards E_{Na} which resulted in the cell interior becoming briefly positive with respect to the extracellular environment (Hodgkin and Katz, 1949). Further consideration of the driving forces for g_{Na} as well as g_K with respect to the active state are presented later in this review.

Additional support for the Na^+ hypothesis of Hodgkin and Katz was provided by radioactive ion flux studies. For example, Keynes (1951) and Keynes and Lewis (1951) demonstrated that net Na^+ entry and a net K^+ loss were associated with the action potential of Sepia axons. These findings were explained on the basis of an increase in Na^+ and K^+ permeability during the action potential. Quantitatively, the observed values of Na^+ and K^+ fluxes were large enough to account for the action

potential in terms of the amount of electric charge transferred across the membrane (Keynes, 1951).

The final precise and detailed explanation of the mechanism underlying the nerve action potential was presented in a series of classical investigations by Hodgkin, Huxley and Katz (1952) and by Hodgkin and Huxley (1952 a-d).

These investigations employed a technique appropriately termed voltage clamp since the axon membrane potential was held or clamped at any desired level by means of a feed-back amplifier. The additional current needed to clamp the membrane potential at any desired level during nerve stimulation could be separated into individual membrane current components. Individual ionic species were associated with each component. For example, depolarizations of 15-110 mV for 10-50 msec duration resulted in a sudden capacity current* followed by an inward current phase which changed direction rapidly into a large and prolonged outward current (Hodgkin et al., 1952). At normal RMP, the inwardly directed current component normally associated with depolarization could be abolished by replacing the external Na^+ with choline; the outward current phase was not affected by this electrolyte modification (Hodgkin and Huxley, 1952a). These results suggested that the directions of the observed currents could be attributed to the initial rise in Na^+ conductance (inward current phase) followed by the prolonged increase of K^+ conductance (outward current phase), these changes being associated with an action potential. Subsequent experiements revealed that Na^+ and K^+ conductances, g_{Na} and g_K, respectively, rise when the

* Capacity current refers to the brief flow of outward current caused by the discharge of the membrane capacitance.

membrane is depolarized and fall when it is repolarized, the changes in g_K being slower and more prolonged than fluxes in g_{Na} (Hodgkin and Huxley, 1952b). Furthermore g_{Na} and g_K were also observed to be a function of RMP; low RMP or maintained depolarization caused an inactivation of g_{Na} which resembled a resting state as the latter is also characterized by low g_{Na}. Thus, at high membrane potentials, an inactivation of g_{Na} appears to be absent. This situation is characterized by optimal g_{Na} in response to supramaximal stimuli; at low RMP g_{Na} is diminished or its inactivation approaches completion (Hodgkin and Huxley, 1952c).

During an action potential, increases in both g_{Na} and g_K occur as a direct result of membrane permeability alterations. Individual ionic species move across the membrane in response to their electrochemical gradient; the driving force for each ionic species is dependent, at least in part, on the equilibrium potential (E) for the ion under consideration. Thus, Na^+ first moves into the cell in accordance with E_{Na}. As the rising phase of the action potential approaches, but never reaches, E_{Na} the g_{Na} is progressively diminishing while g_K is steadily increasing. Throughout this period of inactivation of g_{Na}, the membrane is maximally depolarized and refractory to further stimuli. The increase in g_K causes a net K^+ efflux and corresponds to membrane repolarization in accordance with E_K (Hodgkin and Huxley, 1952d).

A series of equations have been derived to predict action potential pattern and kinetics on the basis of ionic conductances and currents, equilibrium and resting potentials, and various rate constants (Hodgkin and Huxley, 1952d).

In general, the action potential mechanisms described here for squid axon are applicable to muscle cells. However, the RMP of muscle is usually equivalent to E_K while nerve cell E_K is more negative than its RMP (Katz, 1966). Hence, the greater driving force for repolarization of the nerve membrane accounts for the falling phase of the action potential terminating in an after-hyperpolarization; in the case of the muscle, the falling phase of the action potential terminates at a more positive value (close to E_K) than the falling phase of the nerve spike; hence, the absence of after-hyperpolarization in the case of the muscle action potential (Hodgkin, 1958).

Evidence for the Cholinergic Nature of Junctional Transmission

The chemical mechanism of the transfer of impulse from nerve to muscle has been approached from various investigative directions. For example, neurophysiological experiments have revealed that junctional transmission cannot be electrical; this finding adds indirect support to the concept of neurotransmitter involvement. Radioactive tracer techniques, on the other hand, were useful in following the movements of transmitter precursors while electron microscopy revealed probable neurotransmitter storage sites. Hence, this review section concerns not merely the neurochemical evidence for cholinergic neuromyal transmission. Rather, the following is an integration of anatomical, biochemical, neurophysiological and pharmacological evidence which led to the discovery and assertion that ACh was the neurotransmitter at the neuromuscular junction.

Since the early 1900's, overwhelming evidence implicated the functional presence of a transmitter substance in several organs. In 1901, Hunt demonstrated that an aqueous extract of suprarenal glands contained a compound which lowered blood pressure. This substance yielded choline upon decomposition. Later experiments confirmed that the acetate ester of choline was 1,000 times more effective as a vasodepressor than choline (Hunt and Taveau, 1906). These findings indicated that ACh might be the active substance.

The concept of ACh as the transmitter at the neuromyal junction evolved slowly as evidence of several investigators accumulated. Exposure of skeletal muscle to the antagonistic actions of nicotine and curare demonstrated the chemoceptive nature of "chemical" binding sites which initiated or prevented muscle contraction; in fact, Langley (1909) proposed the presence of a "receptive substance" at or on nerve terminals and/or the muscle itself. Release of an active substance by stimulating a nerve was demonstrated by the classical heart perfusion experiments of Loewi (1921). He showed that vagal stimulation released into the perfusate a substance capable of slowing heart rate. The active agent was later identified as ACh by Dale and Dudley (1929) who first isolated ACh from spleen extracts. Dale et al. (1936) subsequently demonstrated that the stimulation of motor nerve fibers to perfused skeletal muscle caused the appearance of ACh in the venous fluid. Furthermore, upon stimulation of denervated muscle, ACh did not appear in the fluid. These and prior contributions enabled Dale et al. (1936) to suggest that the functional link in the transmission between nerve and muscle was indeed ACh. More recent investigations demonstrated ACh release in the absence of nerve stimulation; thus,

there is spontaneous transmitter release at quiescent nerve-muscle junctions (Fatt and Katz, 1950; del Castillo and Katz, 1956; Burke, 1957; Straughan, 1960; Krnjevic and Straughan, 1964).

The concept of cholinergic transmission was also supported by the findings related to the presence of enzymes responsible for the synthesis of ACh. Nachmansohn and Machado (1943) stated that ACh was synthesized within the nerve terminal from choline and acetate in the presence of the enzyme choline acetylase. Acetate from glucose metabolism and coenzyme A (CoA) were required for the synthesis of acetyl coenzyme A; this substance served as the immediate source of acetate for the synthesis of ACh (Berg, 1956 a-b). Choline sources included plasma choline and choline derived from phospholipid breakdown, in addition to choline derived from the enzymatic and extracellular degradation of ACh following nerve terminal release (MacIntosh and Collier, 1976). This degradation results in formation of choline and the uptake of choline was shown to be carrier mediated. This mechanism has been demonstrated in muscle cells (Renkin, 1961; Potter, 1970), squid axons (Hodgkin and Martin, 1965), ganglia (Perry, 1953; Mac Intosh et al. 1956, 1958) and at various other sites including CNS neurons, erythrocytes, etc. Pharmacological evidence supporting the concept of the reuptake of choline for the synthesis of ACh includes the demonstration of the inhibitory actions of hemicholinium at the neuromuscular junction. This competitive inhibitor of choline uptake caused delayed muscle paralysis resulting from the depletion of nerve terminal ACh following repetitive motor nerve stimulation (Schueler, 1960). The hemicholinium structure was shown to contain two choline-like moieties (Schueler, 1960).

Specific molecular sites within the motor nerve terminal at which the synthesis of ACh occurs have not as yet been defined. However, synaptic vesicles are considered to be storage sites for ACh prior to its release (Jones and Kwanbunbumpen, 1970b). The story of synaptic vesicles begins with the demonstration by means of electron microscopy of their presence in prejunctional terminals (De Robertis and Bennett, 1955); it was estimated that frog nerve terminals contained about 3×10^6 vesicles per terminal (Birks et al, 1960). Furthermore, vesicle-rich fractions from cerebral synaptosomes and electric organ were shown to contain high ACh concentrations (Whittaker and Dowdall, 1973). The indirect stimulation of the rat phrenic nerve preparation for 2-4 minutes resulted in a significant reduction in the volume of synaptic vesicles (Jones and Kwanbunbumpen, 1970 a-b). Heuser and Reese (1973) then pointed out that the synaptic vesicles which normally lie close to the terminal axolemmal membrane disappear during stimulation while pits or indentations in the membrane develop. Such changes are consistent with the concept that ACh is discharged by the fusion of vesicles with the terminal membrane and subsequent exocytosis.

Compartmentalization of ACh into several nerve terminal pools was studied largely by cell fractionation procedures applied to brain homogenates (Crossland and Slater, 1968; Slater, 1971). In the case of peripheral nervous tissue, radioactive tracer techniques and physiological procedures which focus on ACh content and transmitter release from intact neurons were applied. Potter (1970) demonstrated that following the stimulation of rat nerve-diaphragm preparations in the presence of labeled choline, [^{14}C] ACh appeared at a fast rate; however, some 20% of

the ACh became labeled at a slower rate. This percentage of transmitter termed "depot store" continued being present within the terminal throughout prolonged stimulation in the presence of hemicholinium; the other 80% of the transmitter, termed readily releasable store, originated from newly synthesized transmitter (Potter, 1970). A similar situation was observed in the superior cervical ganglion (Birks and MacIntosh, 1961).

Neurophysiological evidence also supported the concept of separate transmitter storage pools. High frequency stimulation of nerve-muscle preparations resulted in an initial rapid step-wise decline ("run down") of the amplitudes of the first 3-7 endplate potentials (EPPs). The decline was followed by a plateau, as uniform, lower amplitude EPPs appeared (Elmqvist and Quastel, 1965b; Martin, 1966; Ginsborg and Jenkinson, 1976). This phenomenon suggests that as the readily releasable ACh store is depleted, a second storage or "depot" supply may be mobilized in order to avert total transmitter depletion. Furthermore, EPP amplitude decline following repetitive nerve stimulation (trains) could not be attributed to diminished quantal size (Elmqvist and Quastel, 1965b), decreased postjunctional receptor sensitivity (Otsuka et al.,1962) or to a decrease in presynaptic action potential amplitude (Takeuchi and Takeuchi, 1962).

All this evidence supports the notion of chemical cholinergic mediation of neuromyal transmission. Several neurophysiological and neuroanatomical observations also support the concept of neurotransmitter release at the neuromyal junction by providing evidence which is not consistent with the concept of electrical transmission. For example, individual action potentials propagating through a motor nerve fiber can also be recorded at the muscle fiber innervated by that nerve; however, a consistent delay of 0.5 to 0.8 msec occurs in the transfer of

signal from nerve terminal to muscle cell (Katz and Miledi, 1965 a-b). In addition, electron micrographs revealed the presence of a synaptic cleft at the neuromuscular junction (De Robertis and Bennett, 1955) having an estimated width of 150-250 Å. Subsequent calculations taking into consideration muscle fiber diameters, current pulse, synaptic surface resistance and synaptic gap distance revealed that electrical transmission across a frog neuromuscular junction is not possible (Katz, 1966). As a matter of fact, electrical transmission could account for an alteration of muscle membrane potential by only a negligible subthreshold amount of $< 1\ \mu V$. The above observations taken collectively argue strongly in favor of an electrical and anatomical discontinuity between nerve and muscle.

Electrophysiological Aspects of Neuromuscular Transmission

The electronegativity of the muscle endplate regions resulting from evoked liberation of nerve terminal ACh was initially recorded with crude silver extracellular electrodes (Cowan, 1936; Eccles and O'Connor, 1939). In subsequent experiments where preparations were immobilized by d-Tc local depolarization of muscle fibers termed endplate potential acted as local catelectrotonic potential with a decrement of about 50%-75% per mm (Eccles et al., 1941). Katz (1942) added that a reduction of RMP at a cathode situated at an endplate was associated with an increase in ion permeability observed as an impedance loss; the progressive depolarization and hypothesized resistance breakdown was similar to that observed during the initiation of a nerve action potential. Kuffler (1943) then reaffirmed that ACh depolarized only the regions of the muscle fiber proximal to the neuromyal junction. These findings in conjunction with the Na^+ requirement for the integrity of neuromyal responses led Fatt (1950) to hypothesize

that ACh induced depolarization was related to increased Na^+ permeability at the endplate.

The successful introduction of intracellular recording by means of a glass microelectrode by Ling and Gerard (1949) led to more sensitive and precise electrophysiological measurements of the transmission at the neuromyal junction by Fatt and Katz (1951). This important procedural contribution resulted in a dramatic increase in our knowledge of neuromuscular transmission. Investigators could now distinguish between two forms of transmitter release at the neuromuscular junction. Spontaneous release was unrelated to motor nerve stimulation while evoked release occurred in response to induced nerve impulses. Both types of release exhibited comparable as well as contrasting properties.

1) Spontaneous Release; General Properties.

Insertion of glass capillary tube microelectrodes into frog muscle fiber endplate regions revealed spontaneous subthreshold electrical events not present elsewhere within the muscle fiber (Fatt and Katz, 1950). These events were random, each having a rapid rise and slow decay characteristic of an endplate potential; hence, they were termed miniature endplate potentials (MEPPs). The origin of MEPPs was attributed to the random release of discrete packets of ACh from the motor nerve terminal (Burke, 1957). According to the quantal hypothesis put forward by del Castillo and Katz (1956) a MEPP or quantum was considered to be the smallest unit of release; each quantum was estimated to contain approximately 10^4 molecules of ACh and each prejunctional synaptic vesicle was thought to store an individual quantum (Hubbard and Jones, 1973). This hypothesis was supported in part by earlier experiments which employed low Ca^{++}/high Mg^{++} concentrations in the extracellular medium (del Castillo and

Engbaeck, 1954). Perfusion of the preparation with this modified Krebs solution gradually reduced the quantity of ACh released from the nerve terminal. As a result, the EPP amplitude declined in a series of steps characteristic of the dropping out of quantal units; ultimately, with release being sufficiently depressed, EPPs approximated MEPPs in amplitude and duration. This finding suggested that EPPs were made up of a large number of units (quanta), each unit being equivalent to a MEPP (a single quantum).

MEPP amplitudes were shown to be normally distributed with a mean between 0.1 to 0.5 mV; this value is 1/100 of the average EPP amplitude. Katz and Thesleff (1957) stressed the constancy of quantal size and assumed that discrete quantal units and accompanying conductance changes were identical at the same endplate. However, these investigators observed a 10 fold variation in MEPP amplitude when MEPPs were recorded from different cells and concluded that input membrane resistance was inversely proportional to fiber size; the smaller fibers having greater input resistance exhibited MEPPs of larger amplitude (Katz and Thesleff, 1957). These conclusions were later supported by the results of the voltage clamp investigations of Takeuchi and Takeuchi (1960a): in accordance with Ohms law, relatively small but similar endplate currents corresponded to large EPPs in muscle fibers of high effective resistance and to small EPPs in fibers of lower resistance. A similar relation was presumed to exist between MEPP amplitudes, associated miniature endplate currents and muscle fiber resistance (Takeuchi and Takeuchi, 1960a). Hence, variation in muscle fiber diameters account largely for the noticeable range in MEPP amplitudes when data obtained from several different cells are pooled.

Significant variations of MEPP amplitude were also reported at single endplates. Liley (1957) recorded giant MEPPs that were several times the size of an average MEPP. Giant MEPPs, while few in number, occurred more frequently than would be expected on the random release concept. Giant MEPPs were postulated to arise by the "release of preformed multiquantal aggregates of transmitter substance" (Liley, 1957).

At the other extreme was the appearance of 0.3 to 0.6 mV subminiature EPPs recorded from mouse diaphragm (Kriebel et al., 1976). These minute potentials were not affected by temperature or Ca^{++} concentration changes, contrary to MEPPs. Subminiature EPPs were thought to arise from the release of a quantal subunit (Kriebel et al., 1976) or from an abnormal packet of ACh (Kriebel and Gross, 1974). Alterations of MEPP amplitude could be reflective of changes in the prejunctional quantal size. However, post-junctional changes of postsynaptic membrane resistance, capacitance, RMP or equilibrium potential were more commonly associated with MEPP amplitude variation (Takeuchi and Takeuchi, 1960a; Hubbard et al., 1969a).

From one cell to another, MEPP frequencies exhibited a variation of even greater magnitude than the variation of amplitudes. The frequency ranged from 0.1 to 100 H_z; most frequencies ranged between 1 to 10 $sec.^{-1}$ Any alteration of MEPP frequency always implicated a nerve terminal mechanism (Katz, 1962; Hubbard et al, 1969a). First, nerve terminal polarization alters spontaneous discharge rate whereas muscle cell polarization was without effect on MEPP frequency (del Castillo and Katz, 1954b). Second, MEPPs disappear following motor nerve transection or nerve terminal destruction. The same may be accomplished pharmacologically.

Hemicholinium interferes with the uptake and synthesis of nerve terminal ACh; hence, terminal depletion following repetitive stimulation results in the elimination of MEPPs as well as EPPs (Elmqvist and Quastel, 1965a).

Several procedures or agents also exhibited selective actions on spontaneous discharge rate with few or no effects on MEPP amplitude. For example, MEPP frequency was significantly increased by: stretching the muscle (Fatt and Katz, 1952), increasing temperature (Fatt and Katz, 1952; Hoffmann et al., 1966; Hubbard et al., 1971), elevating osmotic pressure (Fatt and Katz, 1952; Liley, 1956a) or by depolarizing the nerve terminal (del Castillo and Katz, 1954b; Liley, 1956a; Hubbard and Willis, 1962). Ion concentration adjustments also promoted a more rapid spontaneous discharge; a lowered Na^+ concentration in the presence of raised K^+ concentration significantly increased MEPP release. An increase in exogenously applied Ca^{++} or the presence of compounds which elevate Ca^{++} levels consistently increased MEPP frequency (Mambrini and Benoit, 1963; Hubbard et al. 1968a; Goldberg and Singer, 1969; Shinnick, 1974; Alnaes and Rahamimoff, 1975; Kriebel and Gross, 1974; Kriebel et al., 1976). Similarly, the effects of MIX on MEPP frequency suggest the involvement of Ca^{++}; this is further considered in the Discussion section.

2) The Statistical Aspects of Spontaneous Release.

In general, a series of random events reflect a Poisson or exponential distribution if each event is totally independent and thus unaffected by the presence or absence of prior similar events. Furthermore, the events should not occur simultaneously but be distinctly separate

from one another (Hubbard et al., 1969a). In a Poisson distribution of MEPP amplitudes, the probability of the occurrence of a MEPP of a given amplitude should not change significantly over a period of time so long as the data were obtained over similar non-overlapping time intervals.

Still another criterion for a Poisson distribution states that the variance* of a number of events (or intervals) is equal to the mean of all the events (or intervals) occurring over a given period of time (Hubbard and Jones, 1973).

The statistical expression of probability for an event in accordance with Poisson distribution is based upon the following formula:

$$P_{(k)} = e^{-m} m^k/k! \qquad \text{(Gage and Hubbard, 1965)}$$

In this equation the probability (P) of k events occurring within a specific time period is determined by the mean number of total events (m) occurring over the total period of time under consideration. The incidence (n) of a specific number of events (k) in a total population N is:

$$n = N \cdot p_{(k)}$$

From this equation the expected incidence of events reflecting a Poisson distribution can be calculated and compared to the observed incidence of events. A chi square test is employed to test the significance of agreement between the observed distribution and the expected Poisson distribution.

* The variance (V) of a population is a statistical measure of variability equal to the standard deviation squared $(SD)^2$. Variance can be computed by obtaining the deviation of each item from the mean $(x - \mu)$, squaring them $(x - \mu)^2$, summing up all the squared deviations in the population $\Sigma (x - \mu)^2$, and dividing by the total number of items N. Thus, $V = \Sigma (x - \mu)^2/N$. x is the value of each item and μ is the mean value of the items.

Fatt and Katz (1952) first demonstrated that both amplitude and frequency distributions of an 800 MEPP histogram fit an exponential distribution curve characteristic of a random series of events. This observation agreed with a subsequent statistical analysis which characterized MEPP release as closely fitting a Poisson distribution (Gage and Hubbard, 1965; Hubbard et al., 1969a; Rothshenker and Rahamimoff, 1970).

The alterations of experimental conditions by nerve terminal depolarization (Auerbach, 1971) or by increasing the bathing medium Ca^{++} concentration from 2.8 mM to 15.0 mM (Rothschenker and Rahamimoff, 1970) have resulted in MEPP frequency distributions differing from Poisson distribution. However, Cohen et al. (1974b) demonstrated no change in release statistics following a comparable Ca^{++} increase. On the other hand, recent evidence has shown that MEPPs recorded in some experiments in which physiological conditions are duplicated do not reflect a Poisson distribution. While spontaneous release generally meets some of the criteria for a Poisson distribution, other criteria may not be met (Hubbard and Jones, 1973). There may be a deviation from the equality of the variance of the interval between MEPPs and the mean of this interval. Hubbard and Jones (1973) stated that:

> The observed transmitter release may be the pooled output of a finite number of transmitter release sites, each of which release quanta in a random phasic manner or in a manner more ordered than Poisson.

Several procedures and observations led Cohen et al. (1974 a, b, c) to consider Poisson distribution of MEPPs as inappropriate. They used much larger MEPP samples than those included in previous studies. Statistical methods more sensitive to deviations from Poisson were also incorporated and they reported the occurrence of simultaneous events or giant MEPPs.

Altogether, they found evidence indicating that release of one quantum enhances the release of a second one (drag effect), although Hubbard and Jones (1973) found no evidence for the drag effect. Further evidence against non-Poisson distribution of MEPPs included the observation of accentuated MEPP bursts in some preparations (Kriebel et al., 1975; Cohen et al., 1974 a-c). Kriebel et al. (1976) also reported that manipulation of experimental conditions did not affect synchronously released subminiature EPPs whereas normal MEPP distributions were altered.

Current evidence regarding the nature of spontaneous release is, thus, gradually accumulating in favor of a more ordered or perhaps synchronous process which is contrary to a Poisson distribution. The application of more refined investigative and statistical techniques have contributed to the re-evaluation of MEPPs. However, a non-Poisson MEPP distribution may yet be considered as the exception to the rule and resolution of this controversy appears, at this point, to rely on future investigations.

3) Evoked Release; Electrical Properties and Ionic Aspects.

An endplate potential (EPP) is a local depolarization generated at the endplate when a nerve action potential and subsequent Ca^{++} influx (Katz and Miledi, 1965c) invading the nerve terminal triggers a synchronous multiquantal release of transmitter. The free diffusible transmitter combines with the postjunctional receptor sites and the ensuing increase in Na^{+} and K^{+}, but not Cl^{-}, conductance, initiates the ionic currents responsible for the endplate potential itself (Takeuchi and Takeuchi, 1960b). Should this depolarization reach a certain critical threshold, a non-decremental muscle fiber action potential is initiated and propagated,

resulting in muscle contraction. Consequently, the EPP originating prejunctionally and recorded at the subsynaptic endplate region reflects the "chemical" continuity of impulse transmission between the pre and postjunctional elements separated by the synaptic cleft.

Successful intracellular recording of potentials resulting from evoked release at the neuromuscular junction requires that muscle movement be eliminated or greatly reduced. Four methods are commonly employed to achieve this end. The competitive blockade of postjunctional ACh receptors by the addition of sufficient d-tubocurarine reduced EPP amplitudes below the threshold for muscle spike activation (Eccles et al., 1941). Addition of a high Mg^{++}/low Ca^{++} salt concentration to the bathing medium results in a reduction of ACh release causing a decline of EPP amplitude or a failure of release altogether (del Castillo and Engbaeck, 1954); the amount of ACh liberated in the modified, high Mg^{++}/low Ca^{++} Krebs solution varies directly with the Ca^{++} and inversely with Mg^{++} concentration (del Castillo and Katz, 1954a), and can be regulated so as to provide a recordable EPP. The cut fiber technique of Barstad and Lilleheil (1968) requires the transection of muscle fibers along with contractile filaments on each side of the endplate. This procedure depolarizes the fibers by -20 to -35 mV and prevents the conduction of muscle action potentials. Finally, the exposure of muscle to hypertonic ethylene glycol (Sevcik and Narahashi, 1972) results in the destruction of the sarcoplasmic T tubule system; consequently, excitation-contraction uncoupling is achieved.

Following pharmacological, mechanical or toxic immobilization of muscle movement, EPPs could be easily recorded intracellularly. The

potentials were displayed as monophasic waves which were rapidly attenuated as the depolarization moved away from the endplate. When recorded from a "focally" placed electrode, a single EPP usually exhibits a rise time of 1 msec. or less and declines to one half its amplitude in 2 msec. or less (Fatt and Katz, 1951).

The relation Q = CV, where C is the capacitance and V the voltage across the capacitance, denotes the quantity of electric charge (Q) removed from the fiber surface (Hubbard et al. 1969a). Charge transfer is useful in the determination of transmitter depolarizing power and net transport of univalent ions across the endplate. The number of moles of the ion moved across the membrane (n) is derived using a modification of the above equation. Thus:

$$n = CV/F$$

where F is Faraday's constant (10^5). Hence, 3.2×10^{-3} volts cm passed through a 1 cm fiber having a capacity of 2.45×10^{-7} F/cm^2 was shown to have a charge transfer for a subthreshold EPP of 8×10^{-10} coulombs. This charge corresponds to a net ionic flux of at least 8×10^{-15} moles (Fatt and Katz, 1951).

By using the voltage clamp technique, individual ionic currents of an EPP could be explained in a manner similar to that previously described for the nerve action potential. From the Nernst equation, the E_{Na} and E_K were calculated at +50 mV and -99 mV, respectively. Takeuchi and Takeuchi (1959) determined the membrane potential at which the EPP was nullified. This value was -15 mV for frog sartorious muscle and was termed the equilibrium potential of the EPP (E_{EPP}). More precisely, E_{EPP} represented the "membrane potential at which ACh caused neither

hyperpolarization nor depolarization since the sums of currents carried by the ions affected by ACh became zero" (Ginsborg and Jenkinson, 1976). The ionic current, in the case of Na (I_{Na}), was dependent on the conductance of Na^+ (Δg_{Na}) multiplied by the driving force for that particular ion designated as RMP - E_{Na}. Thus, $I_{Na} = \Delta g_{Na}$ (RMP - E_{Na}). Similarly for K, $I_K = \Delta g_K$ (RMP - E_K). The total current (EPC) representing an activated endplate is:

$$I_{Na} + I_K = \Delta g_{Na} (RMP - E_{Na}) + \Delta g_K (RMP - E_K)$$

At E_{EPP}, $I_{Na} + I_K = 0$

By algebraic manipulation $\Delta g_{Na} / \Delta g_K = (E_K - E_{EPP})/(E_{EPP} - E_{Na})$

Substitution of experimentally determined values for E_{EPP}, E_K, and E_{Na} revealed that $\Delta g_{Na} / \Delta g_K = 1.29$ (Takeuchi and Takeuchi, 1960b). Ionic conductances could be altered by the presence of either ACh or d-Tc at the endplate. However, the ratio of $\Delta g_{Na} / \Delta g_K$ remained constant (Takeuchi and Takeuchi, 1960b).

In addition to Na^+ and K^+, Ca^{++} is also involved postjunctionally. Although Na^+ and K^+ fluxes represent the ionic basis of the EPP, Ca^{++} is known as a modulating influence on the EPP. Although this divalent cation contributes insignificantly to EPC, an increase in Ca^{++} concentration from 2 mM to 30 mM selectively reduced g_{Na} change produced by ACh. As a result, EPC amplitude was reduced to 72% of control at a membrane potential of -100 mV (Takeuchi, 1963). This selective influence of Ca^{++} on g_{Na} suggests that the postjunctional membrane consists of separate Na^+ and K^+ channels.

Additional pharmacological evidence supporting the concept of such channels were made possible by the use of toxins and a blocking agent; tetrodotoxin and saxitoxin were observed to prevent voltage dependent changes in g_{Na} while tetraethylammonium blocked g_K (Adrian et al. 1970; Hille, 1970; Kao, 1966; Stanfield, 1970).

4) Evoked Release: Quantal Aspects.

del Castillo and Katz (1954a) demonstrated that endplate potentials can be reduced to a unit identical in size and shape to a MEPP; this can be accomplished by raising the Mg^{++} or lowering the Ca^{++} concentration of the perfusate. An analysis of such reduced EPPs indicated that the variation in the number of units in a long series of EPP responses followed a Poisson distribution. This finding is predictable since the sum of any number of Poisson variates is also a Poisson variate: the Poisson variate being MEPPs or quanta and the sum being the total number of MEPPs (quanta) contained in several EPPs. Consequently, the number of quanta contained in several EPPs during any defined interval should fit a Poisson distribution (Hubbard et al. 1969a).

A single EPP has been estimated to contain between 100 to 200 quanta of ACh (Hubbard and Wilson, 1973) and this accumulation of quanta per EPP was appropriately termed the quantal content (m) of the EPP. The quantal content may vary considerably from one EPP to the next even at the same endplate. The concept of m value means that each nerve action potential is capable of releasing several quanta, each one being identical to an average MEPP in size, from a fixed population of n number of units. Hence, n was designated as the readily releasable store. However, the average

probability (p) of any single quantum being released upon stimulation is also partially responsible for the determination of m. In order for release to comply with a Poisson distribution, p must remain constant from one stimulus to the next and should have a value of < 0.2 (Johnson and Wernig, 1971). However,

> It is not unreasonable to suppose that from stimulus to stimulus n would itself be replenished according to Poissonian statistics, and then even for large values of p the release would also follow a Poisson distribution. (Vere-Jones, 1966)

The relation of p and n to quantal content is expressed as m = n x p, m being quantal content, n - readily releasable stores and p - probability of release. Three commonly used experimental procedures are currently employed in the calculation of m.

Direct Method:

The direct method for measuring m is based on the assumption that an evoked multiquantal release (EPP) is composed of individual quanta equivalent in size to the mean MEPP. Hence:

$$m = \frac{\overline{\text{EPP amplitude}}}{\text{MEPP amplitude}}$$

Direct determination of m is valid only when MEPP and EPP amplitudes are recorded from the same endplate. Although this method has proven to be accurate in the measurement of m, its application to d-tubocurarine treated preparations is difficult since MEPP amplitudes are usually depressed and cannot be readily distinguished from the level of baseline noise.

Variance Method:

The variance method of quantal analysis relies on the application of high frequency (50-200/sec) supramaximal stimuli delivered to the motor nerve for 0.5 to 2 seconds. This procedure results in a "train" of EPPs characterized by the head (first 5-7 EPPs) and the tail (remaining EPPs) of the train. The head EPPs show a gradual amplitude reduction attributed to transmitter depletion. The tail EPPs on the other hand indicate a "leveling off" of amplitudes explained by neurotransmitter mobilization keeping pace with release. Although tail EPPs reveal a random amplitude fluctuation, the train tail represents a steady or constant state. Under such constant conditions the numbers of quanta per EPP vary according to Poisson statistics. Since quantal analysis equations, in this case, are based upon events which fit a Poisson distribution curve, any events characterized by a progressive change are not suitable for variance analysis. For example, the "non-constant" condition characterized by the regression of the train head amplitudes cannot be used in the estimation of m. The progressive decline of these EPPs reflects the initial depletion of transmitter and is not due to the statistical variation in the release process. Consequently, only EPP amplitudes of the train tail are suitable for variance analysis.

In accordance with Poisson statistics the variance of the EPP = $\overline{EPP}$ = SD^2, where $\overline{EPP}$ is the mean EPP amplitude and SD is the standard deviation. Thus,

$$SD = \sqrt{\text{variance EPP}}$$

Accordingly, the coefficient of variation (CV):

$$= \frac{\sqrt{\text{variance EPP}}}{\overline{EPP}}$$

and by substitution of SD for $\sqrt{\text{variance EPP}}$:

$$CV = {}^{SD}/_{\overline{EPP}}$$

By definition the quantal content of the EPP is:

$$m = {}^{1}/_{CV^2} \qquad \text{(del Castillo and Katz, 1954c)}$$

The variance method is an indirect means of estimation and is subject to more error than the direct measurement. However, its accuracy can be improved if a large number of EPPs (about 200) are used in the estimate. Boyd and Martin (1956) employed the variance method in the analysis of two junctions of cat tenuissimus muscle; m value estimates were 220 and 310 quanta, respectively.

Failure Method:

The failure method of estimating m is restricted to experiments in which transmitter release has been inhibited by decreasing Ca^{++} and/or raising the Mg^{++} concentration of the Krebs perfusate. This method of estimation makes use of extremely small EPPs (1 to 3 quanta) since EPP failures occur only after extensive depression of release.

Calculation of m is based on the property of Poisson statistics that a sample variate, in this case m, having a value equal to 0, 1, 2, 3, etc., occurs as an expected proportion of the total number of events. More precisely, the probability of occurrence of an EPP with x quanta is given by the equation:

$$P_x = \frac{e^{-m} m^x}{x!}$$

where m is the mean number of quanta liberated by an impulse or:

$$m = \frac{\overline{\text{EPP amplitude}}}{\overline{\text{MEPP amplitude}}}$$

The probability of occurrence of an EPP with 0 quanta (a failure) is given by the relation:

$$P = e^{-m}$$

In agreement with Poisson theorem the quantal content can be estimated as:

$$m = \ln \frac{\text{number of impulses}}{\text{number of failures}}$$

This calculation was described by del Castillo and Katz (1954b). Assuming a Poisson distribution, the determination of m by the failure method demonstrates good agreement with the variance estimate of m so that:

$$m = \frac{\overline{\text{EPP amplitude}}}{\overline{\text{MEPP amplitude}}} = \ln \frac{\text{number of impulses}}{\text{number of failures}}$$

In 1971, Johnson and Wernig described the criteria for a Poisson versus binomial distribution and the means of distinguishing between the two. Their approach was applicable to EPP failures. Hence, the use of their logic was also applied to EPP failure experiments reported in this dissertation; a binomial distribution was confirmed. For the purpose of avoiding repetition the reader is referred to the Results section for a detailed analysis and summary of distribution type and the application of appropriate equations necessary to calculate quantal parameters on the basis of binomial distribution.

The Endplate Acetylcholine Receptor

To deliver ACh to the muscle endplate the neurotransmitter can be ejected from a micropipette by iontophoresis. ACh can also be released from the nerve terminal whether by spontaneous release, electrical stimulation of the nerve or by artificial depolaration of the nerve terminal. By whatever means sufficient, ACh is subsequently brought in contact with the subsynaptic area, the ensuing depolarization is directly attributable to the combination of neurotransmitter with postjunctional receptor sites.

In general, a:

> Receptor is a specialized component of a cell possibly a macromolecule, with which an endogenous molecule or pharmacological agent interacts to initiate a chain of events leading to a response. (Shirachi et al. 1973).

Receptors possess chemorecognitive properties making them highly specific for certain agonists or antagonists; thus, receptors have little affinity for non-specific agents.

The concept of a neurotransmitter receptor developed at the beginning of the 20th Century in conjunction with the idea of chemical transmission. Langley (1909) demonstrated that neither nicotine nor curare, at various concentrations were able to prevent the contraction of muscle induced by direct stimulation. Yet, both compounds were mutually antagonistic in the absence of stimulation; nicotine evoked muscle contraction and curare blocked these actions of nicotine. Langley reasoned that the mutual antagonism of nicotine and curare suggested a competition by each for a specific site since the contractile mechanism in muscle was not involved. This site was termed the receptive substance since its occupation could initiate or inhibit the contractile response (Langley, 1909).

Neurotransmitter receptors at the neuromuscular junction are appropriately termed nicotinic since they can be activated by nicotine as well as ACh. The chemical specificity of these receptors are thus characterized through the actions of antagonists (d-Tc, gallamine, pancuronium, succinylcholine, decamethonium).

Since the formulation of the receptor concept, many aspects of receptor investigation have become highly technical and specialized areas of endeavor. For example, contemporary pharmacological evidence for receptor distribution and sensitivity of the muscle is largely the result of ACh iontophoresis investigation. Initial experiments employing microiontophoretic techniques revealed that ACh was most effective in depolarizing muscle cells when applied to the endplate region (Nastuk, 1953). del Castillo and Katz (1955b) later indicated that intracellular injection of ACh was ineffective and concluded that transmitter receptors were extracellularly located.

A subsequent study expressed the sensitivity of ACh potential as the ratio of the amplitude response in mV to the amount of ejection charge passed through the micropipette in nC (10^{-9} coulomb) (Peper and McMahan, 1972). These investigators employed Nomarski optics for optimal endplate localization and quantified endplate sensitivity relative to non-endplate regions. Thus, while the endplate revealed a peak chemosensitivity up to 1900 mV/nC, a ten fold decline was noted 5 to 10 μm from the terminal's edge. To make receptors even more accessible, snake muscles were treated with collagenase to allow the separation of motor nerve synaptic boutons from underlying endplates (Kuffler and Yoshikami, 1975). Exposed subsynaptic membrane craters directly beneath removed terminals

exhibited sensitivities as high as 500 mV/nC while 2 μm away the sensitivity was 50 times lower. Additionally, potential time courses induced by ACh iontophoresis at subsynaptic craters approaches those of the synaptic potentials set up by ACh released by the nerve. Enzymatic removal of connective tissue and motor nerve endings thus permitted positioning of the micropipette at the site normally occupied by prejunctional membranes; furthermore, the findings illustrated the striking contrast in endplate versus non-endplate ACh sensitivity. The highest sensitivity, reflective of the highest concentration, of heretofore unreachable ACh receptors was located directly beneath the motor nerve terminal with the postjunctional indentations.

The location and distribution of muscle ACh receptors were investigated by means of radioactively labeled neuromuscular blocking drugs. Tagged receptors could be easily quantified by the use of serial section autoradiography (Waser, 1960, 1967). These studies employed labeled d-Tc and related blocking drugs. However, the use of such reversible and relatively non-specific antagonists resulted in marked inconsistencies due to non-specific binding and to the difficulty in determining when binding saturation was reached. The discovery of irreversible antagonists including polypeptide toxins or snake venoms eliminated much of the experimental error previously encountered.

Snake venom toxins, isolated and purified by Chang and Lee (1963) are currently popular in receptor isolation studies. Both α-bungarotoxin and cobra venom toxins are examples of antagonists which irreversibly block neuromyal transmission by combining with and inactivating, ACh receptors (Lee, 1972). Several investigators have supported the

concept of the toxin-receptor binding by pointing out that toxin binding is more concentrated at the endplate region than elsewhere (Miledi and Potter, 1971; Barnard et al., 1971; Berg et al. 1972; Porter et al., 1973; Anderson and Cohen, 1974). Denervation experiments yielded additional pertinent data. Skeletal muscle denervation resulted in increased ACh sensitivity along the entire lengths of muscle fibers which was parallel with the increased spread in the number of ACh receptors (Lunt et al., 1971); thus following denervation α-bungarotoxin was no longer bound mainly to the endplate region of denervated preparations, and its uptake by the whole muscle was 30 fold as compared to the uptake by innervated muscle (Hartzell and Fambrough, 1972; Chang et al., 1973; Colquhoun et al., 1974).

In yet other experiments, d-Tc was shown to be incapable of competitively reducing by more than 50% the amount of α-bungarotoxin bound after a given time (Porter et al., 1973; Albuquerque et al. 1973). Albuquerque et al. (1973) further demonstrated that perhydrohistrionicotoxin (from the Columbia arrow-poison frog) blocks neuromuscular transmission but also prevents the binding of α-bungarotoxin only partially. Yet the combination of d-tubocurarine and perhydrohistrionicotoxin given together prevented the binding of α-bungarotoxin almost completely. These results suggested that the ACh receptor contained two independent binding sites, one being sensitive to d-tubocurarine and the other to perhydrohistrionicotoxin. The latter site was considered to be the ionic conductance modulator or ionophore which mediates permeability change in response to receptor activation (Albuquerque et al., 1973; Barnard et al., 1975).

α-Bungarotoxin was also used for the isolation and identification of the receptor substance from muscle and receptor dense electric organ tissue. Various separative techniques including labeled toxin receptor combination, affinity chromatography and density gradient centrifugation were employed to this end (Changeaux et al., 1970; Miledi et al., 1971; Miledi and Potter, 1971).

Molecular characterization of the ACh receptor from Torpedo tissue was derived from extensive binding, separation and purification procedures (Miledi et al., 1971; Meunier et al., 1972). These authors described the ACh receptor as a protein subunit of low density owing to its association with lipid molecules; the estimated molecular weight of each subunit was calculated to be about 48,000. A separate analysis of the receptor dense electric organ from the ray Torpedo characterized the ACh receptor as a complex protein composed of six macromolecular subunits each having a molecular weight of 40,000 (Raftery, 1973). Additional information concerning ACh receptors and their isolation and characterization was reviewed by O'Brien et al. (1972), Landowne et al. (1975), Rang (1975) and Triggle and Triggle (1976).

The Localization and Function of Acetylcholinesterase at the Neuromuscular Junction

In the aftermath of his identification of "Vagustoff" as ACh, Loewi (1921), noted that following release, transmitter action was quite rapidly terminated. This occurrence suggested the involvement of a hydrolyzing enzyme; this was suggested independently by Dale. A cholinesterase enzyme or enzymes were subsequently implicated in the biochemical degradation of transmitter since the presence of these enzymes was

demonstrated in almost all animal tissues (Plattner and Hinter, 1930). Later studies revealed that cholinesterase activity, determined manometrically, was maximal in the muscle region containing nerve endings (Marnay and Nachmansohn, 1938) and that following denervation cholinesterase activity at the endplates persisted (Couteau and Nachmansohn, 1940); this activity gradually dissipated with time (Snell and McIntyre, 1956; Elias, 1972).

Initial histochemical studies of acetylcholinesterase by Koelle and Friedenwald (1949), Koelle (1950, 1951) and Couteaux and Taxi (1951, 1952) also localized the highest concentrations of enzyme in the zone of motor innervation. More recent investigations in which histochemical techniques were combined with electron microscopy revealed that more intense staining for AChE occurs in the secondary subneural membrane folds of sarcoplasmic origin (Koelle, 1963; Csillik and Knyihar, 1968). These findings reinforce the concept that the highest concentration of AChE is present postjunctionally in contrast to prejunctional loci.

The Koelle method relied on the following series of reactions for enzyme visualization:

$$\text{Acetylthiocholine (substrate)} \longrightarrow \text{thiocholine} + Ci^{++}$$

$$\longrightarrow \text{copperthiocholine} \longrightarrow CuS\downarrow$$

However, histochemical studies of cholinesterase were made difficult by the presence of several esterases. Besides AChE located primarily in nervous tissue, endplates and red blood cells, there is butyryl cholinesterase (BuChE or pseudocholinesterase) found mainly in serum and viscera but also at the muscle; other forms of the enzyme were also distinguished (Usdin, 1970). Consequently, discriminative techniques to distinguish

between both types became necessary.

In one case selective staining for AChE involved a modification of the Koelle method requiring pH manipulation (Lewis, 1961). Another method called for the incubation of sections with selective BuChE inhibitors such as a low 0.1 μ mol/l concentration of diisopropyl-flourophosphate (DFP) which inhibited BuChE more readily than AChE. Koelle (1963) used differential BuChE-AChE inhibitors and substrates; altogether, information as to AChE was obtained by comparing staining of sections incubated with substrate, sections treated before and after the addition of DFP to eliminate BuChE, and sections treated, before addition of substrate, with the anticholinesterase physostigmine which inhibits both AChE and BuChE (cf. Hobbiger, 1976). More detailed information concerning histochemical localization of AChE can be found in reviews by Couteau (1955), Koelle (1963) and Koelle et al. (1967).

The results of pharmacological experiments have supported the significant role of AChE in relation to neuromuscular transmission. EPPs and MEPPs recorded from skeletal muscle were increased in amplitude and duration following cholinesterase inhibition by various drugs such as neostigmine (Eccles et al., 1942; Fatt and Katz, 1951, 1952; Magleby and Stevens, 1972; Katz and Miledi, 1973b). The observed alterations of junctional potentials were not related to increased release of transmitter since the anti ChE edrophonium, 5×10^{-5} M did not significantly increase quantal content (Ferry and Marshall, 1971). A method for estimating AChE inhibition could be derived on the basis of the magnitude of EPP prolongation by anti ChE's (Ferry and Marshall, 1971). The non-competitive organophosphorus inhibitor echothiopate shows pseudo

first order reaction kinetics for AChE inhibition. Maximum inhibition was assumed when 99% of the physiologically active AChE was inhibited. The degree of inhibition at intermediate EPP prolongation times was consequently calculated from the pseudo first order reaction kinetic equation.

The iontophoresis or perfusion of ACh or analogues at concentrations subeffective for transient depolarizations are not totally without effect. For example, Katz and Miledi (1970, 1971, 1973 a, b, c) have shown that the level of baseline fluctuation termed acetylcholine "noise" resulted from cholinergic agonist-receptor interaction. More precisely, such noise was defined as contributing to the:

> seemingly steady depolarization and was made up of an enormous number of components each resulting from the transient opening of an 'elementary' ion channel; the noise thus results from the fluctuations in the number of channels open from moment to moment. (Ginsborg and Jenkinson, 1976).

The analysis of noise fluctuation allows an estimate to be made of the elementary (current/voltage) conductance changes in terms of the "life-time of the ion channels" (Ginsborg and Jenkinson, 1976). Hence, time constant decays for various cholinergic agonists may provide some criteria for determining efficacy of drugs acting at the same level of receptor occupancy. Katz and Miledi (1973b) stated that AChE did not alter the magnitude or duration of the elementary voltage change resulting from ACh receptor combination. Their experiments indicated that AChE did not hydrolyze ACh already combined with the receptor; rather the enzyme acts to hydrolyze the free transmitter. Thus, following significant enzyme inhibition, the

potentiation of transmission was attributable to repeated collisions of ACh with receptors. In the presence of AChE inhibition, such repeated transmitter collisions also impeded the simple diffusion of ACh out of the synaptic cleft (Katz and Miledi, 1973b) contributing to the characteristic EPP amplitude and time course facilitation.

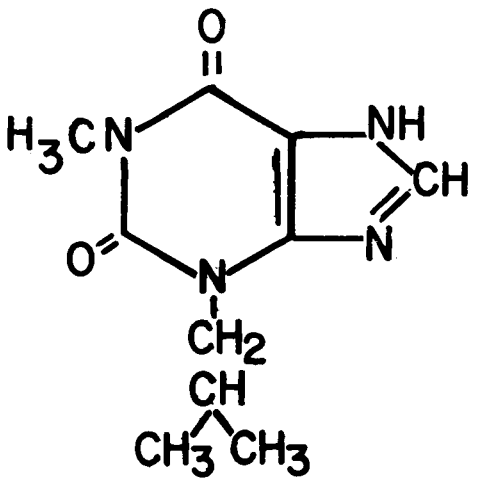
These results indicate clearly that the role of AChE is to cleave ACh at the neuromyal junction enzymatically, thus terminating its action, in order to check excessive receptor activation following release. Some choline is formed via the action of ChE. Of the choline thus formed more than one half is eventually taken up by the nerve terminal for resynthesis, storage and subsequent release (Potter, 1970).

Actions of Methylxanthines and Methylisobutylxanthine on Neuromuscular Transmission

1) Molecular Structure of Xanthines.

In general, references to methylxanthines commonly pertain to five compounds. Caffeine is described as 1,3,7- trimethylxanthine. The 1,3-dimethyl and 1,7- dimethyl xanthines are more commonly distinguished as theophylline and theobromine, respectively. In addition, two molecules of theophylline have been combined with one molecule of ethylenediamine in the synthesis of aminophylline® (Searle). Aminophylline is often substituted for theophylline in experiments as the former is more soluble than theophylline. Another xanthine, oxtriphylline is the theophylline salt of choline.

The structure of the recently synthesized compound, 1-methyl-3-isobutylxanthine (MIX), the subject of this dissertation, differs from theophylline only in the substitution of an isobutyl group at the 3-methyl position (Figure 1).



METHYL ISOBUTYL XANTHINE

Figure 1. Chemical structure of 1-methyl-3-isobutyl xanthine.

2) Methylxanthines (other than MIX).

Xanthines act on most systems of the living organism. For example, xanthines have been shown to: enhance the strength of skeletal muscle contraction; relax smooth muscle; stimulate the CNS cortex and the respiratory, vasomotor and vagal centers; exhibit positive inotropic and chronotropic actions at the heart; augment gastric secretions; increase basal metabolism, and cause diuretic action (Goodman and Gilman, 1975). However, the following review of methylxanthines will be limited to their effects on neuromuscular transmission.

Initial investigations of methylxanthine actions lacked the precision and technical capabilities to demonstrate specific neuromyal sites of drug actions. Hence, the effects of methylxanthines were first reported with respect to muscle contraction. In 1945 Torda and Wolf indicated that at concentrations of 1.1 to 5.5 mM caffeine, theophylline or theobromine increased the magnitude of frog rectus muscle contraction when excitation was induced by either ACh, 50 μg/100cc or K^+, 20 mM. The same compounds were also shown to be effective against fatigue* or partial curarization of the cat quadriceps muscle (Huidobro, 1945). Later experiments confirmed a weak anticurare action of theophylline and also revealed that muscle contraction in response to direct stimulation was potentiated by theophylline following total curarization (Goutier, 1949). A review by Sandow (1965) provided a detailed explanation for the caffeine related increase of the force of muscle contraction. This phenomenon was explained as a caffeine induced lowering of the

* Fatigue was demonstrated as decreasing muscle twitch response to indirect stimulation at high frequencies (60-400 Hz).

threshold for mechanical activation due to enhanced Ca^{++} release from the sarcoplasmic reticulum.

Caffeine was also reported to slightly prolong the muscle action potential; this prolonged the time during which the membrane potential was above the threshold for the mechanical response (Sandow et al., 1964). The area under the action potential curve and above the threshold for mechanical activation was shown to be proportional to the duration of the mechanically effective period. Thus, an increase in the area under the action potential curve resulted from a lowered threshold for the mechanical response and from an increase in the duration of the action potential itself. Hence, a prolonged active state or prolonged mechanically effective period resulted. Sandow (1965) proposed such a dual effect as a basis for caffeine action on muscle contraction.

Walther (1962) found that caffeine and theophylline were more effective in raising the force of contraction of muscle fatigued by high frequency stimulation (8 H_z) of the phrenic nerve-diaphragm and less effective in reversing fatigue due to low frequency stimulation (0.3 H_z) over several hours. Increased K^+ levels potentiated methylxanthine actions (Walther, 1961). These and prior observations allowed Walther (1962) to suggest that caffeine and theophylline increased the sensitivity of the motor endplate.

In addition to the methylxanthine induced increase of spontaneous release in frog sartorius (Mambrini and Benoit, 1963; Onodera, 1973), frog cutaneous pectoris (Duncan and Statham, 1977), rat diaphragm (Elmqvist and Feldman, 1965; Goldberg and Singer, 1969; Hofmann, 1969)

and cat tenuissimus (Shinnick, 1974). A number of additional findings also support a prejunctional site of action. Hofmann (1969) demonstrated that caffeine (2 mM) stimulated transmitter replenishment (mobilization) but quantal content of the first few EPPs was not increased in the rat diaphragm. Wilson (1973) found that 1.0 mM caffeine not only increased mobilization rate but also increased quantal content and probability of release in the isolated cut fiber rat diaphragm preparation. Similar rat diaphragm data were also obtained with 1.0 mM concentrations of theophylline and aminophylline although the latter compound did not significantly increase readily releasable stores (Wilson, 1974a). However, Shinnick (1974) reported no change of releasable stores in d-Tc blocked cat muscle; a significant decrease in releasable stores occurred in cut fiber preparations in response to 0.9 mM aminophylline.

Further signs of prejunctional facilitation by aminophylline 0.9 mM, included the elevation of quantal content which may also contribute, at least in part, to the observed increase in EPP amplitude (Shinnick, 1974).

The earlier consideration of xanthine related increase in receptor sensitivity (Walther, 1962) was re-examined by Shinnick et al. (1976) on the basis of her studies of aminophylline action at the neuromyal junction. Her findings were as follows. Aminophylline potentiated the responses to iontophoretically applied ACh. Cholinesterase inhibition may have been involved since a carbachol depolarization response was potentiated to a lesser degree than the ACh response. Aminophylline also decreased membrane resistance due to an increase in the ionic permeability; this was consistent with aminophylline-induced resting

membrane depolarization. A shift of the E_{EPP} to a more positive level was also recorded in the presence of aminophylline; the shift contributed to an increased driving force for the EPP, $RMP-E_{EPP}$, which would account, at least in part, for the subsequent increase in EPP amplitude (Shinnick, 1974; Shinnick-Gallagher and Jacobs, 1976).

3) The Methylxanthine-Cyclic Nucleotide Link.

Experiments by Butcher and Sutherland (1962) and Sutherland et al. (1968) resulted in the definition of cyclic nucleotides as metabolic mediators or second messengers following their discovery in a wide range of cell types. cAMP is synthesized from ATP in the presence of the enzyme adenyl cyclase while the enzyme phosphodiesterase is responsible for the degradation of cAMP to physiologically inactive 5' AMP:

$$\text{ATP} \xrightarrow[\text{Cyclase}]{\text{Adenyl}} \text{Cyclic AMP} \xrightarrow[\text{Phosphodiesterase}]{\text{cAMP}} \text{5' AMP}$$

There is considerable evidence supporting the involvement of cAMP in neuromuscular transmission as a result of these findings:

a. Both epinephrine and methylxanthines elevate cAMP by two different pathways: epinephrine activates adenyl cyclase to facilitate the production of cAMP from ATP (Jenkinson et al., 1968), while methylxanthines inhibit phosphodiesterase to prevent enzymatic degradation of cAMP (Breckenridge, 1970). The common effect of both facilitators at the neuromuscular junction results in both an augmentation of transmitter release (Goldberg and Singer, 1969) and an increase in the force of muscle contraction (Breckenridge et al., 1967). Similar findings by others support the concept of a xanthine related increase in cAMP as

the basis for enhanced junctional transmission (Wilson and DeVilley, 1973; Wilson, 1974a). Furthermore, β-adrenergic blocking agents such as propranolol, which inhibit an increase in cAMP levels also block epinephrine induced increase in twitch tension (Breckenridge et al., 1967; Varagic and Zugic, 1971). However, propranolol does not block the xanthine facilitation presumably because xanthines act beyond the adenyl cyclase step (β-receptor site) (Haugarrd and Hess, 1966).

b. In the presence of ATP, cAMP activates a phosphorylase which in turn catalyzes the breakdown of glycogen to glucose-6-phosphate (Hornbrook and Brody, 1963 a, b). Addition of 125-500 μg of exogenous glucose-6-phosphate to each ml. of perfusate produced exactly the same effects on the isolated nerve-diaphragm preparation as xanthine derivatives and catecholamines, that is, it potentiated the response of the diaphragm to indirect and direct stimulation and it exhibited an anti-curare action (Varagic and Zugic, 1971).

c. Presumably, because of an inability of cAMP to enter cells, the perfusion of this compound alone demonstrated little or no effect (Varagic and Zugic, 1971). In contrast, the application of dibutyryl cAMP mimicked the actions of either epinephrine or various methyl-xanthines on neuromuscular transmission (Goldberg and Singer, 1969). The increased effectiveness of the dibutyryl derivative accounted for the actions characteristic of elevated cAMP concentrations including increases in EPP amplitude, quantal content, and MEPP frequency (Goldberg and Singer, 1969; Wilson, 1974a).

d. Sodium flouride, an activator of adenylate cyclase, and dibutyryl cAMP both increased stimulus bound repetitive activity (SBR) recorded from the cat tibial nerve which innervates the soleus muscle (Dretchen et al., 1976; Standaert et al., 1976 a, b). In addition, these investigators stated that theophylline (100 μg/kg) induced SBR in the motor axons and potentiated and prolonged the effects of dibutyryl cAMP and sodium fluoride.

e. Dibutyryl cAMP (0.25 mM) in conjunction with theophylline (0.5 mM) potentiated the depolarization caused by iontophoretic application of ACh to rat diaphragm muscle (Ewald, 1976 a, b).

f. It was postulated that cAMP is involved in the generation of ganglionic potentials. In the superior cervical ganglion, post-ganglionic levels of cAMP were increased in response to preganglionic stimulation (Greengard and Kebabian, 1974). This increase in cyclic nucleotide was postulated to be triggered by the release of dopamine from ganglionic interneurons; dopamine was stated to activate adenylate cyclase which in turn enhanced synthesis of cAMP. In turn, increased cAMP levels were responsible for a hyperpolarization of the postganglionic membrane resulting in the slow inhibitory post synaptic potential (sIPSP). Greengard and Kebabian further hypothesized that a concomitant but undefined ionic permeability change was linked to cAMP related activation of protein kinase which caused phosphorylation of a specific substrate protein in the membrane; the phosphorylated protein was thought to result in the alteration of membrane permeability and the observed sIPSP (Greengard and Kebabian, 1974). Additionally, Greengard

reported that phosphodiesterase inhibitors, including theophylline, potentiated both cAMP accumulation and the sIPSP following either preganglionic stimulation or incubation of the preparation with dopamine (McAfee and Greengard, 1972; Kalix et al., 1974; Greengard and Kebabian, 1974). In the present context, these results provide a mechanism for the action of methylxanthines via the cyclic nucleotide related effects. However, insofar as the ganglion is concerned, other workers could not duplicate Greengard's results (Dun and Karczmar 1977, 1978; Gallagher and Shinnick-Gallagher, 1977; Kuba and Nishi, 1976).

g. Although cAMP enhanced $^{45}Ca^{++}$ efflux from the fly salivary gland (Prince, 1972), a cAMP-Ca^{++} functional link was less likely in the nerve terminal; Wilson (1974a) indicated that elevating Ca^{++} resulted in increased probability of release whereas elevating dibutyryl cAMP did not. Regardless of their relationship, both Ca^{++} and cAMP mechanisms must be considered in any xanthine investigation. Possible functional links between xanthines, Ca^{++} and/or cAMP in terms of neuromuscular transmission are presented in more detail in the Discussion Section. The connection between Ca^{++} and cAMP in various tissues is also the subject of a review by Berridge (1975).

4) Methylxanthines and Myasthenia Gravis.

The use of methylxanthines in the management of myasthenia gravis is pertinent in the present context. This condition was described as a failure of the muscle to respond to indirect stimulation but not to direct stimulation (Jolly, 1895). Initially, a defect in neuromuscular transmission was implicated. Evidence in support of this

defect theory was presented by Elmqvist et al. (1964) who demonstrated MEPPs of depressed amplitude in myasthenic muscle. The reduced quantal units were suggested to result from diminished synthesis of ACh. However, the mechanism was not related to a hemicholinium-like block of choline uptake since the addition of choline to the perfusate did not restore MEPP amplitude to normal. More recently, studies employing labeled α-bungarotoxin revealed that myasthenic muscle showed a 70% to 89% reduction in acetylcholine receptors per neuromuscular junction as compared with control muscles (Drachman et al. 1976, Drachman 1978 a, b). This receptor deficit may be explained by an auto immune reaction as a result of the discovery that many myasthenic patients produced serum antibodies directed against skeletal muscle (Strauss et al., 1960) or against the acetylcholine receptor itself (Mittag et al., 1976).

Myasthenic muscle weakness is generally alleviated by anticholinesterase therapy (Flacke,1934). However, a muscle biopsy preparation from one patient, refractory to several anticholinesterases showed a marked reduction of weakness after application of aminophylline. The compound "restored strength to near normal; muscle contractions fused into tetanus at a frequency of 30/sec, whereas, during the control period, the muscle failed to respond to 3/sec stimuli" (Jacobs et al.,1971). Subsequent experiments on myasthenic muscle biopsy preparations reaffirmed that aminophylline augmented twitch height and that it possessed anti-fatigue actions (Jacobs et al.,1973). Clinical use of aminophylline resulted in prohibitive side effects. On the basis of the aminophylline findings by Jacobs et al. (1971, 1973) and in an effort

to diminish side effects, oxtriphylline, a choline salt of theophylline, was administered to five patients (Brumlik et al.,1973). In general, oxtriphylline not only increased the level of muscle strength when used alone, but it potentiated the effect of anticholinesterases and augmented the beneficial effect of corticotropin. Oxtriphylline was generally effective in patients resistant to anticholinesterase medication even though the compound alleviated the symptoms of myasthenia gravis only partially (Brumlik et al.,1973).

5) 1-methyl-3-isobutylxanthine (MIX).

The majority of investigators employing MIX were primarily interested in the potent phosphodiesterase inhibitory action of the compound. Beavo et al. (1970) compared the biochemical effectiveness of 64 xanthine derivatives, including MIX. In this investigation MIX exhibited a potency fifteen times greater than theophylline in its lipolytic effects on epididymal fat cells and by its ability to inhibit the enzyme phosphodiesterase. Collier et al. (1976) noted a ten fold difference in the potencies of MIX and theophylline in various tissues. For example, approximate mM values for 25% inhibition (IC 25) of cAMP phosphodiesterase from rat brain homogenates were 1.5 mM for theophylline but only 0.15 mM for MIX: for the human lung, the IC 25 values were 0.14 mM for theophylline and 0.024 mM for MIX. In another investigation, MIX was two hundred times more potent than aminophylline in the cAMP mediated secretion of alpha-ecdysome, a secretion from the prothoracic glands of the tobacco hornworm (Vedeckis et al.,1976). The inhibition of phosphodiesterase by MIX results in increased tissue levels of cAMP (Beavo et al.,1970). The

cyclic nucleotide acts as a mediator or regulator of metabolic processes (Sutherland et al. 1968).

Exposure of isolated pancreatic islet cells to 0.1-10 mM MIX caused consistent increase in cAMP levels (Montague and Cook, 1971; Rabinovitch et al., 1976; Kempen et al., 1977 a, b). A 0.1 mM MIX concentration not only increased cAMP levels, but potentiated a glucose related increase of insulin release from the pancreatic islets of normal and diabetic Chinese hamsters (Rabinovitch et al., 1976). This effect on insulin suggests possible clinical relevance to diabetics.

Exposure of rat liver preparations to MIX resulted in a more rapid, greater and sustained increase in cAMP than the exposure to aminophylline (Costa et al., 1975). This effect was associated with enhanced protein kinase activation and a significant potentiation of phenylephrine induction of glycogenolysis and gluconeogenesis (Cherrington et al. 1976).

Numerous investigations have demonstrated that MIX is not cell type selective in its enzyme inhibitory actions since this xanthine elevated cAMP levels in rat pituitaries (Schofield and McPherson, 1974; Schofield et al. 1974; Azhar and Menon, 1977), quartered rat adrenal glands (Peytremann et al. 1973), rat heart (Keely et al. 1975; Corbin et al. 1977), rabbit intestinal smooth muscle (Schubert et al. 1975; McKenzie et al. 1977b), transitional epithelium of the rabbit urinary bladder (Chalapowski, 1975), and various reproduction system cells including sea urchin sperm (Garbers and Hardman, 1976), rat testis interstitial cells (Williams et al., 1976; Cooke et al., 1976a, 1976b), and ovarian tissue of the chick embryo (Teng and Teng, 1977).

In addition, MIX-related increases in cAMP levels were observed in several tumor cells; these included human epidermoid carcinoma (HEp-2) cells (Kelly and Butcher, 1975) and cultured neuroblastoma cells (Schultz and Hamprecht, 1973) which also exhibited an elevated rate of glycogenolysis (Passonneau and Crites, 1976). MIX also potentiated prostaglandin E_1 induced elevation of cAMP levels in neuroblastoma cells (Hamprecht, 1973), and enhanced the augmentation by arachidonic acid of the cAMP levels in cell cultures obtained from C3H mouse mammary tumors (Burnstein, 1977). Leichtling et al. (1976) observed that following incubation of human astrocytoma cells in low concentrations of prostaglandin E_1 (0.003-0.1 mM) the ability of the cells to increase cAMP was lost following more than one challenge of catecholamines or prostaglandin E_1; this phenomenon was totally reversed by MIX, and it was suggested that MIX alleviated desensitization of the cell to prostaglandin E_1.

Cyclic guanosine 3', 5'-monophosphate (cGMP) levels are also influenced by MIX. Thus, MIX further increased cGMP concentration differences between the relatively high levels of the cyclic nucleotide in rat liver hepatomas compared to lower levels in surrounding control liver slices (DeRubertis and Craven, 1977). MIX also inhibited hydrolysis of cGMP by fraction II (a cGMP phosphodiesterase) with an I_{50} value of 3 μM for heart and liver and 50 μM for cerebrum (Kakiuchi, 1975). In rat parotid tissue slices MIX enhanced the ability of low (otherwise ineffective) concentrations of carbachol to increase cGMP accumulation (Butcher et al., 1976), and several xanthines including MIX inhibited both cAMP and cGMP related phosphodiesterase of the rat

anterior pituitary (Azhor and Menon, 1977). In the thyroid glands of the horse and dog MIX blocked the activation of cAMP hydrolysis by cGMP, as cGMP was thought to facilitate cAMP breakdown through a direct activation of phosphodiesterase in the absence of MIX (Erneaux et al., 1977). An earlier study also demonstrated that MIX and tri-iodothyronine have similar phosphodiesterase inhibitory activity (Van Inwegan et al., 1975). Thus, in addition to direct cAMP and cGMP phosphodiesterase inhibition, MIX may also function to prevent cGMP activation of cAMP phosphodiesterase, at least in some endocrine cells.

In addition to inhibition, MIX has effects that may not be related to the elevation of cyclic nucleotides. The sporulation of Clostridium perfringens was increased in the presence of MIX (Sacks and Thompson, 1975). Application of MIX to B-16 melanoma cells stimulated maturation, retarded the release of sialoglycoproteins and elevated melanin content (Banks et al., 1975). MIX was also reported to inhibit the activity of Poly (ADP ribose) polymerase, an agent suggested to be involved with the repression of DNA synthesis and cell proliferation in differentiating cardiac muscle (Claycomb, 1976). In another study MIX induced α-aminoisobutyric acid transport in rat liver parenchymal cell cultures that had been treated with dexamethasone (Pariza et al., 1976).

Additional endocrine interactions include the following: MIX significantly increased insulin secretion and cAMP levels in rat and mouse pancreatic islets of Langerhans which may suggest an insulin related effect on cAMP turnover (Montague and Cook, 1971; Grill et al., 1975; Hellman, 1975, 1976; Hahn and Gottschling, 1976; Haring et al., 1976). In rat anterior pituitary cells MIX stimulated the release of

growth hormone (Mira-Moser, 1976). However, colchicine inhibited the release of growth hormone in bovine anterior pituitary slices (Sheterline et al. 1975) in the presence of MIX (0.1 mM). Prolactin release was also observed in rat pituitary (GH) cells in culture following MIX, 0.03 mM (Dannies et al. 1976). In other investigations MIX doubled testosterone production (Cooke et al., 1976a) and stimulated the release of amylase from rat parotid acinar cells (Kanagasuntheram and Randle, 1976). Corticosterone production by rat adrenals in the absence of ACTH was increased by MIX (Peytremann et al., 1973).

Two distinct actions of MIX in the central nervous system have been reported thus far. In rats treated with several xanthines including MIX, a small dose of naloxone precipitated a quasi-morphine abstinence syndrome (QMAS) that was difficult to distinguish from the true morphine abstinence syndrome (TMAS) evoked by the same dose of naloxone in rats made dependent on morphine (Collier, 1974; Collier et al.,1974; Francis et al., 1975; Collier and Francis, 1976; Collier et al., 1976). Furthermore, the general nature of both TMAS and QMAS may be similar because xanthines also intensify the TMAS (Collier and Francis, 1975; Francis et al., 1975). Collier et al. (1976) found that MIX and naloxone act synergistically and, hence, assumed that each acts at a difference part of a related mechanism. On the basis of the powerful phosphodiesterase inhibitory action of MIX, it may be suggested that the xanthine sensitizes the cyclic nucleotide apparatus to an endogenous substance acting in the same direction as naloxone (Collier et al., 1975, 1976).

Another action of MIX in the CNS is on catecholamine systems. MIX alone or in combination with papaverine potentiated the attenuation of electrical activity responses of the rat caudate nucleus induced by dopamine or cAMP applied microiontophoretically (Siggins et al.,1974). Furthermore, support for the idea that cAMP is the mediator of the increased release of dopamine after nerve stimulation is afforded by the fact that MIX by itself caused a potentiation of the induced release of ^{3}H-dopamine from rat neostriatum brain slices (Westfall et al.,1976).

MIX, when compared to other xanthines, was the most potent compound in potentiating the norepinephrine induced activation of phosphorylase in isolated guinea pig heart (McNeill et al.,1973); MIX increased the overflow of norepinephrine elicited by nerve stimulation and caused a concentration dependent inhibition of splenic perfusion pressure responses to nerve stimulation in the isolated perfused cat spleen (Cubeddu et al., 1975). This latter effect was attributed to the smooth muscle relaxant properties of phosphodiesterase inhibitors and in this case resulted in decreased splenic vascular resistance (Cubeddu et al., 1975). Other smooth muscle investigations indicated that MIX was able to reduce the tone of rabbit duodenal muscle and negated the tone enhancing effect of the hormone analog, 13-norleucine motilin (Schubert et al., 1975).

The ability of MIX to antagonize adenosine actions was dependent on the type of tissue used in the study. For example, MIX was able to antagnonize the adenosine induced accumulation of cAMP levels in brain slices as determined from studies which traced the formation of cAMP

from labeled adenine (Huang et al., 1972). However, at MIX concentrations subeffective for the relaxation of smooth muscle (100 μM), the xanthine was unable to inhibit adenosine induced relaxation of the isolated longitudinal muscle of rabbit small intestine; the same 100 μM dose of theophylline inhibited the adenosine induced relaxation (McKenzie et al.,1977a).

There are no investigations concerning the action of MIX on the skeletal muscle or neuromuscular junction. In contrast to most of the studies cited which employ MIX as a biochemical tool, my investigation deals with MIX related alterations of transmission at the mammalian neuromyal junction.

METHODS

Surgical Procedure

Adult male and non-pregnant female cats weighing 2-5 kg were selected for use in all microelectrode experiments. Animals were anesthetized with intraperitoneal injection of a mixture of α-chloralose, (60 mg/kg) and pentobarbital sodium (5 mg/kg).

The hind leg of the cat was shaved and the skin was incised between the Achilles tendon and the sacral vertebrae to uncover the popliteal space. The skin immediately surrounding the incision was cauterized free from adjoining blood vessels and connective tissue, thus exposing the biceps muscle. This muscle was carefully elevated, bisected with the cautery and folded back in order to expose the underlying tenuissimus muscle. A segment of the tenuissimus muscle, containing a branch of the sciatic nerve inervating it, was ligated proximally and distally and carefully freed from the surrounding connective tissue. The attached bifurcating nerve was also trimmed free of connective tissue; extreme care was exercised in order to prevent severing or damaging the distal segment of the nerve toward its insertion into the tenuissimus muscle. A suitable length of nerve was selected, ligated near its bifurcation from the sciatic nerve, and cut free. The entire nerve-muscle preparation was placed in a perfusion chamber. The tissues were immediately perfused with an oxygenated Krebs-Henseleit solution while adhering fascia was removed using microdissection instruments with the aid of a dissecting microscope.

Perfusion System

Two 500 ml leveling bulb reservoirs contained Krebs solution which was aerated with 95% O_2 and 5% CO_2. The left reservoir was always filled with normal Krebs perfusate while the test drug was dissolved in the solution contained in the right reservoir. Each reservoir was provided with rubber tubing, 3.0 mm I.D., which led to a common inlet where gravity flow rate of either solution could be regulated. Appropriate clips positioned at the two sources of a shared tubing system allowed selective flow of either normal or drug containing solutions. The common inlet terminated in .043 in. (lumen diameter) polyethylene tubing which coursed through a heated condenser used to maintain the perfusate at a constant temperature of 37°C. The segment of tubing leaving the condenser was attached to an 18G needle which served as the perfusion chamber inflow.

The 9.0 x 1.4 cm perfusion chamber (Figure 2) was constructed from plexiglass. The bottom of the chamber contained a layer of translucent Dupont Sylgaard permitting transillumination of the preparation. The muscle could be readily pinned to the Sylgaard layer. Magnification of the preparation up to 107.5x was achieved with an American Optical dissecting microscope.

In order to reduce the recording of excessive stimulus artifact, the nerve stimulation chamber (Figure 2) was isolated from the larger muscle perfusion chamber; the nerve from the tenuissimus was passed through a narrow channel into an expanded chamber containing two bipolar platinum electrodes. This chamber was also used to perfuse the nerve.

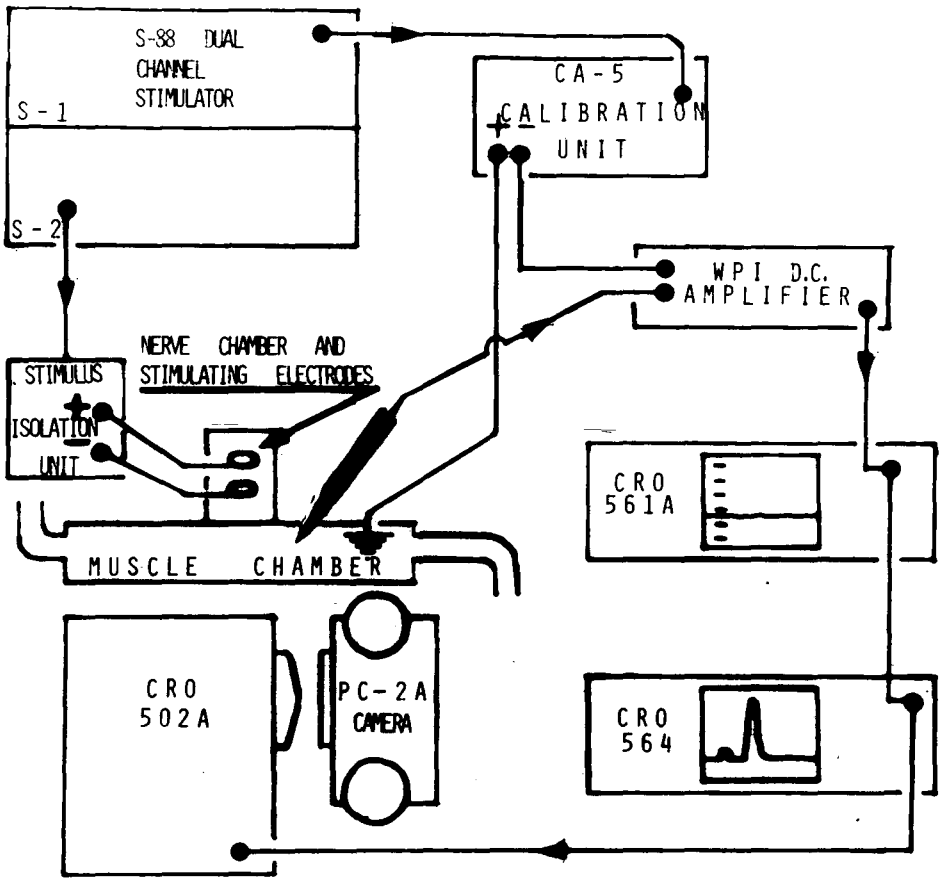


Figure 2. Diagramatic representation of the recording and stimulating circuits used for studying junctional potentials. CRO; cathode ray oscilloscope. See text for further details.

Maximum capacity of the double perfusion chamber was 5.5 ml and the flow rate was maintained at 5.8 ml/min. Thus, complete turnover of chamber volume took place approximately every 57 seconds.

Electrophysiological Recording and Stimulating Apparatus

Microelectrodes were prepared by drawing out glass capillary tubes in a David Kopf vertical electrode puller. Electrode tip resistance was estimated by the rate of ethanol flow into the tip as electrode resistance is inversely proportional to rate of flow; in general, electrodes with flow rates of .010 mm/sec. or less were suitable. "Electrodes prepared in this manner have been shown to have a tip diameter of 0.5 to 1.0 μm and a resistance of 4-25 MΩ " (Shinnick, 1974). Selected electrodes were then completely filled with ethanol under vacuum. The ethanol was displaced with distilled water which was subsequently displaced with 3.0 M KCl.

A 3" length of Ag wire was electroplated with AgCl and inserted into the KCl electrode. The wire extending from the capillary barrel served as the point of contact with the recording circuit. The electrode was then secured to a Narishige micromanipulator to allow precise electrode placement at any selected locus.

The KCl electrode was connected to a WPI DC amplifier which, in turn, was coupled to three oscilloscopes. The first of these oscilloscopes, a Tektronix RM 561A, was calibrated at 20 mV/div to record the resting membrane potential (RMP). The second, Tektronix RM 564 storage oscilloscope was set at a higher amplification to record smaller

junctional potentials. A third, Tektronix 502A, dual beam oscilloscope with attached 35 mm Nikon Koehdon PC-2A Reflex camera was used to obtain permanent photographic records of junctional potentials. A written record of RMP read off the monitor scope, was kept for all experiments.

One channel of a dual channel Grass S-88 stimulator was connected to a Bioelectric CA-5 calibration unit. This arrangement allowed triggered calibration pulses of known amplitude and duration to pass through a lead to the WPI amplifier and be displayed on the recording oscilloscopes (Figure 2). The calibrator also served to certify oscilloscope calibration. A ground electrode from the CA-5 calibration unit to the muscle chamber perfusate completed the recording circuit (Figure 1).

Square wave pulses, of 50-100 μsec. duration, originating from the second channel of the S-88 stimulator were passed through a stimulus isolation unit to the bipolar platinum stimulating electrodes. Where indirect stimulation was required, the threshold voltage was determined and then quadrupled to ensure supramaximal nerve stimulation. Stimulation voltage usually ranged from 20-30 volts. Figure 2 illustrates the stimulation circuit.

Junctional potentials were photographed on Kodak 35 mm film. Following development the film was read in a magnifying viewer. Amplitude and time course determinations were obtained from the accompanying calibration pulses and the data retained for permanent record.

MEPP Recording

Trial and error microelectrode insertions at endplate dense areas of the muscle were attempted in order to locate spontaneous miniature

endplate potentials (MEPPs). MEPPs that exhibited with the greatest amplitude and rise times shorter than 1 msec served as indicators of precise electrode positioning at the endplate (Gallagher, 1972). A MEPP experimental trial was initiated when at the end of a 10 min observation period the resting membrane potentials were stabilized and did not decline from the original values by more than 5 mV.

Most experiments including those concerned with MEPP recordings were divided into three 10 minute periods: control period, drug treatment period and post treatment rinse period. Photographic recordings were made during the final 2 minutes of each cycle. The control cycle was immediately followed with a single drug concentration perfusion for 10 minutes. The post treatment recordings did not necessarily demonstrate complete reversibility of the drug actions; they indicated, however, whether or not there was any return to control conditions representing waning of a drug effect. For MEPP photography the oscilloscope sweep speed was set at either 0.5 cm/sec or 10 cm/sec. Recurrent one-second sweeps were then filmed during the last two minutes of each cycle; the PC2A camera was set in the vertical continuous mode at .5 cm/sec. The sweep speed of 10 cm/sec served to record MEPPs as single deflections of varying size and number per sweep. From this information MEPP amplitude and frequency data were obtained. In order to record MEPP time course the oscilloscope sweep rate was readjusted to 0.5 cm/msec. A representative number of sequential single frame photos were taken during each two minute period. These recordings provided adequate rise time and half decay time information.

MEPP amplitude was reported in mV while frequency was presented in H_Z. All time course data was expressed in milliseconds (msec). Results from all experiments were expressed as means $\pm$ standard error.

MEPP amplitude and frequency distribution histograms for the low and high drug concentrations tested were constructed. The frequency histograms compared each MEPP frequency per second in terms of their percentual incidence with respect to the total sample. Similarly, MEPP amplitudes were plotted in terms of the frequency incidence with respect to the total sample.

EPP Recording

Endplate potentials (EPPs) resulting from indirect supramaximal stimuli were evoked at a frequency of 0.1 H_Z. d-tubocurarine chloride (d-Tc), 7.29 μM, was perfused to inhibit muscle movement during the experiments. MEPP localization by way of random probing in endplate-rich areas was often necessary to locate the EPPs arising within the endplate region. Several microelectrode repositionings were sometimes necessary to insure optimal recordings which yielded control potentials with amplitudes greater than 1.5 mV and rise times* not greater than 1 msec in duration.

EPP recordings were preceded by a five minute observation period during which RMP and EPP amplitudes were monitored as in the case of MEPP experiments. Experimental protocol for acceptable cells included control, treatment and post treatment (rinse) periods, each lasting ten minutes.

EPPs were photographed on sequential single frames with the camera in the vertical mode. The oscilloscope sweep speed was set at

* Time taken for EPP to attain maximum amplitude.

0.5 cm/msec and the trace displaying the calibration pulse and EPP was triggered by the stimulator. EPP parameters from several acceptable experiments, that included the 3 phases of the usual protocol, were grouped and the data expressed as means.

Recording Trains of EPPs for Quantal Analysis

Acceptable EPPs were located as previously described. Muscle immobilization was again achieved by the use of d-Tc. Trains were then obtained by stimulating the preparation indirectly at a rate of 50 Hz for two seconds; intervals of one minute were maintained between consecutive trains. The control, treatment and rinse sequence for trains was identical with those employed for MEPP and EPP experiments.

Recording methods, however, were altered as compared with those described above. A pin point stationary beam was positioned on center screen of the recording oscilloscope. Repetitive EPP "deflections" were then spatially separated by using the camera in the horizontal continuous mode with the film speed adjusted to 20 cm/sec.

Trains were divided into the head (1st 20 EPPs) and the tail (remaining 80 EPPs) for the purpose of analysis. The amplitudes of each EPP together with the observed RMP and the previously determined equilibrium potential of -15 mV for d-Tc blocked muscle (Blaber,1970; Takeuchi and Takeuchi, 1959) were entered into a Digital Lab 8E computer. A computer program (Blaber, 1970) based on the Elmqvist and Quastel (1965b) quantal analysis included steps needed for the calculations of quantal size (q), quantal content of the 1st EPP (m_0), quantal content of the train tail (m_1), readily releasable stores (n) and probability of release (p).

When the EPP amplitude is small ($<$ 3 mV), the number of quantal units are proportional to the EPP amplitude (Martin, 1966). As the EPP amplitude is increased, additional quantal units contribute less to further increments of EPP amplitude. The current underlying the EPP depends on the conductance change as well as on the driving potential ($RP-E_{EPP}$). If the EPP is relatively small (a small percentage of the driving potential) the time course of the synaptic current approaches the time course of the underlying conductance change thus representing a linear response. McLachlan (1978) also stated that resistance to current flow at the synaptic cleft may be appreciable; by reducing the driving force this would reduce non-linear summation. In order to avoid underestimations of quantal content, EPP amplitudes were adjusted by using Martin's (1955) correction factor for non-linear summation of EPP quanta. Martin's correction factor is mathematically expressed as follows:

$$EPP' = \frac{EPP}{(RP- E_{EPP}) - EPP} \times (RP- E_{EPP}) \quad (1)$$

where EPP' is the corrected endplate potential, RP the resting membrane potential, and E_{EPP} the equilibrium potential of the EPP (Martin, 1955).

The correction for non-linear summation of the unit potentials which compose the EPP constituted the initial calculation in the quantal analysis program. Such corrected train tail EPPs were said to vary according to a Poisson distribution curve (Martin, 1955).

The EPP was shown to be composed of individual packets or "quanta" of ACh, each approximately 1/100 of the normal endplate response to a motor nerve impulse (Fatt and Katz, 1952). Subsequent experiments have demonstrated that decreasing the Ca^{++} and/or increasing the Mg^{++} concentrations of the perfusate can result in a stepwise diminution of the EPP until the amplitude response approaches that of a single MEPP (del Castillo and Katz, 1954a). Consequently, the mean MEPP amplitude is considered equivalent to the mean quantal size:

$$\bar{q} = \overline{MEPP} \qquad (2)$$

In the presence of d-Tc, MEPPs could not be recorded simultaneously with trains of EPPs, and the direct measurement of quantal size was not possible in this case. q values could, however, be determined by means of variance analysis of train tail EPPs. On the assumption that these EPPs conformed to a Poisson distribution, the calculation of q is given by the following equation (Edwards and Ikeda, 1962; Elmqvist and Quastel, 1965b):

$$\bar{q} = \frac{\text{variance of EPPs}}{\text{mean EPP}} \qquad (3)$$

Further program design (Blaber, 1970) grouped tail EPPs into blocks for comparative purposes. Blocks consisting of five or ten EPPs were entered into the computer. Corrected EPP amplitude means and variances were calculated for each block and these values were substituted in equation (3) in order to obtain mean quantal size ($\bar{q}$).

Quantal content of the first EPP (m_o) of each train was determined from the following relationship:

$$a = mq \text{ (Brown, 1962)} \qquad (4)$$

By algebraic manipulation:

$$m_o = a/\bar{q} \qquad (5)$$

where a equals the amplitude of the 1st EPP and $\bar{q}$ is the mean quantal size. The same equation also applies to the determination of the mean quantal content of the train tail (m_t). However, in this case, mean EPP amplitude is substituted for "a"; thus:

$$m_t = \frac{\overline{EPP}}{\bar{q}} \qquad (6)$$

The first few EPPs of each train are known to decline in a stepwise fasion (Elmqvist and Quastel, 1965b). This phenomenon was interpreted as a prejunctional depletion of the neurotransmitter ACh and "indicates that the presynaptic store from which transmitter is derived has a limited size, and at first is not replenished as fast as it can be emptied" (Elmqvist and Quastel, 1965b). Consequently, determination of the readily releasable store (n) relies on the slope of the observed EPP "run-off" which is attributed to quantal depletion. The computer program plotted the quantal content of each head EPP (first 4 EPPs) versus the cumulative quantal depletion. The linear relationship of the EPP stepwise decline was assessed by correlation coefficients which compared the first EPP with remaining EPPs of the head of the train. A linear regression plot was included to obtain x and y intercepts. The y intercept signified the quantal content of the first EPP (m_o), while the x

intercept represented the readily releasable store of transmitter (n). Experimental determination of n relies on the regression plot of the first four EPPs since the early decline represents the depletion of the prejunctional store with negligible transmitter mobilization. Mobilization is apparent as the initial EPP decline deviates horizontally or plateaus to form the train tail (Elmqvist and Quastel, 1965b). Values for transmitter mobilization (dm), usually expressed as quanta/msec, could be calculated by dividing the mean train tail quantal content by the stimulation pulse interval (Blaber, 1970).

Quantal content (m), readily releasable stores (n) and probability of release (p) are related as follows (del Castillo and Katz, 1954c):

$$m = np \qquad (7)$$

Probability of release was thus obtained from the ratio of the first EPP quantal content (m_o) to the previously determined readily releasable store (n).

Thus:

$$p = {}^{m_o}/_{n} \qquad (8)$$

Quantal Analysis by Means of EPP Failures

The quantal analysis as carried out by the Elmqvist and Quastel (1965b) EPP train variance method is valid if the transmitter release conforms to Poisson statistics. Poisson distributed release could not be demonstrated on the basis of EPP train data alone. Hence, the method of Johnson and Wernig (1971) which involves the calculation of quantal parameters as well as the quantal distributions for predicted binomial and Poisson release was employed. This method relies

on the presence of EPP failures.

The tenuissimus nerve-muscle preparation was used in EPP failure experiments. Stimulation and recording circuits were identical with those used for MEPPs and EPPs measurement. The preparation was indirectly stimulated at a rate of 10 Hz to provide a sufficient number of EPPs. Recording oscilloscope traces were adjusted to provide recurrent one-second sweeps. Observed EPPs appeared as single deflections of variable magnitude on each recorded sweep. Calibration pulses preceded each EPP. The events were photographed with the camera in the vertical continuous mode at 0.5 cm/sec. Low, 0.98 mM Ca^{++}/high 3.74 mM Mg^{++} Krebs perfusate is known to impair pre-synaptic transmitter release (del Castillo et al.,1954; Katz et al.,1965; Hubbard et al.,1968 a, b). The introduction of this modified Krebs solution results in the absence (failure) of some EPP responses to indirect supramaximal stimuli. Experiments were initiated when observed failure rates stabilized at five to fifty percent over a ten minute observation period. Experimental protocol for collecting failure data was identical with the ten minute period procedure described earlier. The effects of methyl isobutylxanthine (MIX),0.006 mM to 0.5 mM, were tested on the incidence of failures. Failure rates were calculated with respect to 250 stimuli recorded during each control and treatment cycle.

The detailed description of the determination of quantal analysis parameters including quantal content, probability of release and readily releasable stores obtained from EPP failure experiments is included in the Results section. Furthermore, specific equations which related the quantal distributions of a series of EPPs containing failures to either

binomial of Poisson release are presented in conjunction with the Results section.

ACh Perfusion Depolarization

Acetylcholine perfusion experiments were carried out with muscle preparations in contrast to the nerve-muscle preparations employed in other experiments. The muscle was placed in the perfusion chamber, cleaned and securely pinned down as already described. The recording circuit was essentially as described earlier (Figure 3). In addition, a Harvard Apparatus pen recorder calibrated to indicate potential deflections of 1 to 100 mV was connected to the recording oscilloscope. The recorder provided permanent traces of the potentials.

The perfusion system for this experiment was also modified as compared to that described before. There were four leveling bulbs A-D containing respectively:

normal Krebs solution;

10-20 ug/ml ACh dissolved in normal Krebs solution;

MIX, 0.5 mM dissolved in normal Krebs solution; and

10-20 ug/ml ACh and 0.5 mM MIX dissolved in normal Krebs solution.

The perfusion system was arranged so that any one of the four solutions could be selected for perfusion via gravity flow.

The protocol of the ACh depolarization experiments was as follows (cf Figure 3). The microelectrode was first positioned intracellularly at an endplate region of a cell exhibiting suitable resting membrane potential. The preparation was initially perfused with

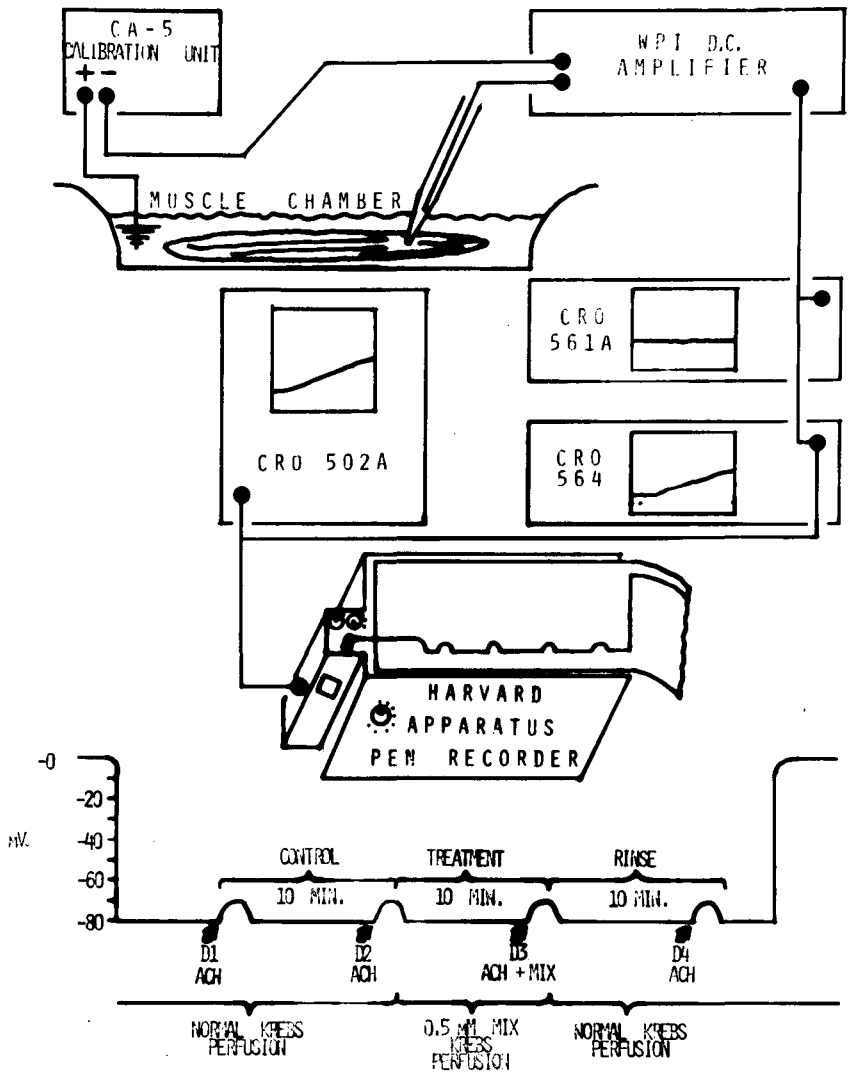


Figure 3. A diagramatic representation of the ACh perfusion depolarization recording circuit (above) and the protocol for a typical experiment (below).

normal oxygenated Krebs solution throughout a short observation period. The experiment was initiated by perfusing the preparation with ACh-Krebs; a 5-15 mV depolarization (D_1) was observed. After the maximal depolarization (D_1) was obtained, an immediate washout perfusion of normal Krebs solution resulted in recovery and served to prevent receptor desensitization. Ten minutes after D_1, a second ACh depolarization (D_2) was induced; D_2 served as a second control and marked the end of the control cycle. Uniform D_1, D_2 responses suggested absence of desensitization; the few preparations that exhibited a progressive decline in response were discarded. Following D_2 the preparation was perfused for 10 minutes with Krebs solution containing MIX (0.5 mM). This treatment period was terminated with another ACh depolarization (D_3) representing the treatment response. The post treatment rinse or treatment recovery period was then initiated by rinsing the preparation for ten minutes. Ten minutes after D_3, a final ACh depolarization (D_4) was obtained. D_4 represented the post treat control response and concluded the experiment (cf Figure 3).

Iontophoretic ACh Potentials

The term iontophoresis is defined in this dissertation as the electrically activated ejection of ACh from a micropipette positioned extracellularly at the muscle endplate. Resulting endplate depolarizations or ACh potentials were then monitored by means of a intracellular recording electrode, located proximally to the endplate. Iontophoresis is a unique means of application as it serves to deliver a discrete concentration of the transmitter (ACh) at a precise locus (the endplate), thus bypassing the prejunctional nerve terminal stores. The iontophoretic method was first described in detail by Nastuk (1953).

Ejection pipettes were prepared as described above; they were filled with 2.5 M ACh Cl. ACh iontophoresis electrodes had tip resistances ranging from 40-100 MΩ. ACh in solution is a positively charged ion. Consequently, administration of a positively charged electrical field to the pipette caused ACh ejection. Reversing the polarity results in retention of ACh in the pipette; the current is appropriately termed braking, retaining or backing current (Del Castillo and Katz 1955b).

Suitable surface cells were obtained by means of locating appropriate MEPPs. An intracellular KCl recording electrode was employed for this purpose. With the exception of a Grass S-48 single channel stimulator to trigger calibration pulses, the recording circuit employed for recording ACh potentials was unchanged from that used to record EPPs (Figure 4).

A second micromanipulator holding the ejection pipette was located opposite the recording electrode. This arrangement allowed optimal extracellular placement of the ACh pipette in close proximity to the intracellularly placed recording electrode tip.

The stimulating circuit included the use of a Grass S-88 dual channel stimulator and 2 SIU-5 stimulus isolation units connected in series. One stimulator channel functioned to deliver the ACh ejection pulse. The other channel supplied a continuous DC backing current to prevent ACh leakage between pulses (Del Castillo and Katz, 1955b; Peper and McMahon, 1972). One stimulus isolation unit lead served as an indifferent electrode and made contact with the muscle chamber perfusate. A lead from the micropipette to the other isolation unit included a 50 MΩ shunt resistor and a 47 KΩ resistor placed across

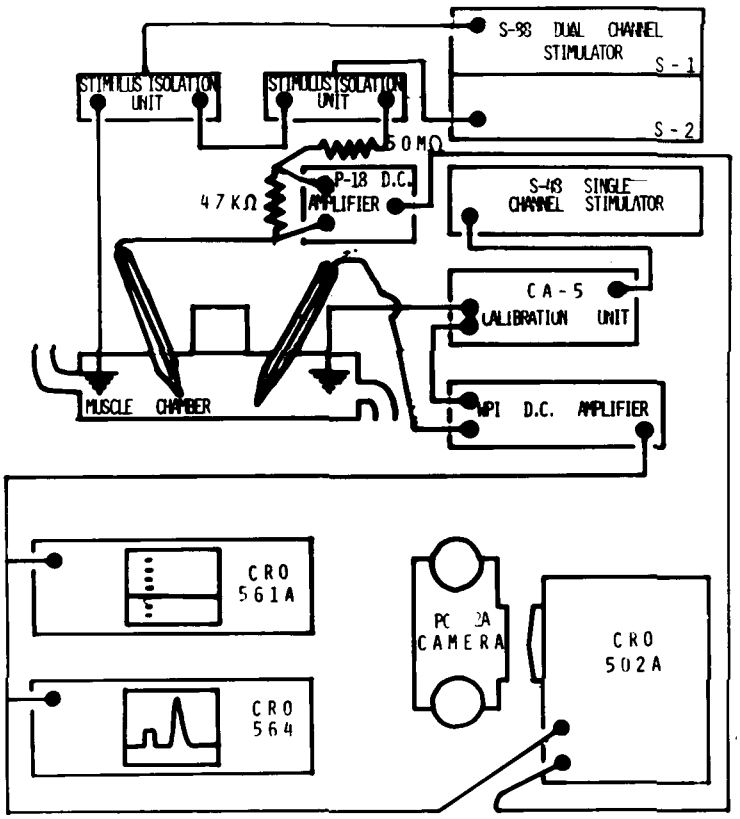


Figure 4. The stimulating and recording circuits employed in microiontophoretic experiments. CRO- cathode ray oscilloscope. See text for further explanation.

the DC amplifier input terminals. The ejection current was then monitored as the voltage drop across the 47 KΩ resistor; a Grass P-18 DC amplifier was employed for this purpose. The current pulse was displayed on the lower beam of the Tektronix 502A dual beam oscilloscope. A diagramatic illustration of the iontophoretic stimulating and recording circuits is shown in Figure 4.

Several difficulties were encountered in the course of iontophoretic experiments. Ejection pipette placement required continuous positional adjustments alternating with ejection pulses; this procedure was needed to obtain suitable iontophoretic potentials prior to the actual experiment. Too, radical pipette repositioning resulted at times in cell impalement. Ejection of excess ACh frequently caused the iontophoretic potential to achieve threshold and result in a twitch; such movements dislodge or damage ejection pipettes or result in damaged or depolarized cells. Desensitization characterized by diminished postjunctional responses to identical iontophoretic pulses constituted yet another problem; this was attributable to unduly frequent ACh ejection pulses or to repeated receptor exposure to excessive ACh concentrations. To avoid desensitization, ACh was applied every 30 seconds.

Several variables influenced the choice of a suitable ejection current; they included the selection of the electrode, the presence of excess connective tissue impeding ACh diffusion onto the receptor target, and the distance between the electrode tip and the receptor. Accordingly, pulse strength and duration had to be frequently varied from one cell to the next or between each electrode placement if

acceptable iontophoretic potentials were to be obtained during the observation and control periods. Ejection currents were approximately 10 nA and 10-70 msec duration. Backing current was adjusted to prevent ACh diffusion from the micropipette between pulses.

Consistent iontophoretic and resting membrane potentials over the 10 min pretreatment control and observation period were used as criteria for selecting acceptable experiments. Furthermore, potentials were acceptable if they were at least 2 mV in amplitude and had a time course no longer than 200 msec. Adjustment of stimulator settings or electrode repositioning were never attempted with an experiment in progress. In the actual experiments, a 0.5 mM MIX dose was employed. The rate of stimulation was 0.5 H_z over the 10 min periods described previously for MEPP and EPP experiments.

Input Resistance of the Muscle Membrane

Experiments performed to measure input or passive membrane resistance (R') made use of two intracellular KCl microelectrodes; one electrode functioned to pass electrical current across the membrane resulting in polarizing electrotonic potentials while the other served to record these potentials. The stimulating and recording circuits were similar to those described in detail by others for cat tenuissimus (Martin, 1959) and frog sartorius preparations (Fatt and Katz, 1951).

A current passing (stimulating) electrode containing KCl was inserted intracellularly at an endplate region within 100 μm of the recording electrode. The insertion of two electrodes into a single cell resulted in a noticeable 10-20 mV decline of resting membrane potential.

The stimulating circuit included a current passing electrode connected in series to a 50 MΩ shunt resistor. The voltage drop across a 47 KΩ resistor, also in series with the electrode, was amplified by means of a Grass P-18 DC amplifier and displayed on the Tektronix 502A dual beam oscilloscope. The voltage drop measurement across the 47 KΩ resistor allowed calculation of the current intensity in accordance with Ohm's law:

$$I = V/R$$

where I is the current in amperes and v the voltage drop across R, the 47 KΩ resistor.

A series of six hyperpolarizing anodal electrotonic potentials of varying intensity having a duration of 60 msec each were filmed during the final two minutes of the pretreatment control period. Comparable potentials were similarly obtained during the MIX treatment and post-treatment cycles.

The relation between depolarizing (cathodic) currents and resulting electrotonic potentials were shown to be non-linear (Katz, 1948). Anodic potentials continued to rise for a shorter time and showed no significant departure from Ohm's Law (Katz, 1948). Hence hyperpolarizing (anelectrotonic) potentials were used in the calculation of membrane resistance. Resistance values were determined as follows. The previously calculated current strength (I) together with the observed anodal electrotonic potential amplitudes (V) were substituted in Ohm's equation to determine the input membrane resistance:

$$R' = V/I$$

The relationship between each observed electrotonic potential and its accompanying current pulse were used for the construction of a current voltage plot. The slopes of these plots thus represented the input membrane resistance and provided a convenient means of comparing control and treatment data. A paired t-test was employed as a check of statistical significance between control and treatment resistance values.

Cholinesterase Assay

Total Cholinesterase activity of cat tenuissimus muscle homogenate, in the presence and absence of MIX, was determined by using the method first described by Ellman et al. (1961). The Ellman colorimetric assay depended on the presence of acetylthiocholine as an enzyme substrate which was enzymatically cleaved to its thiocholine and acetate components. The thiocholine immediately reacted with added dithiobisnitrobenzoate (DTNB) ions to produce yellow 5-thio-2-nitro-benzoic acid anions. Formation of yellow color was measured spectrophotometrically over a selected period of time to serve as a quantitative index of cholinesterase activity.

Ellman et al. (1961) indicated that non-specific esterase may be selectively inhibited by appropriate concentrations of quinidine or DFP in order to determine precise acetylcholinesterase activity. Such selective enzyme inhibition was not attempted in the cat tenuissimus assays for several reasons. First, selective inhibition of non-specific esterase cannot always be accomplished without also inhibiting some acetylcholinesterase. Second, according to Barnard et al. (1971), the difference between acetylcholinesterase and total cholinesterase in

mammalian muscle were rather insignificant, characterized by respective active sites per endplate of 2.2×10^7 and 4.6×10^7 in rat diaphragm muscle. Hence, reference to acetylcholinesterase inhibition (rather than to total cholinesterase) while technically incorrect, is nevertheless appropriate on the basis of acetylcholinesterase concentration predominance. Finally, the purpose of this assay was not to distinguish the relative contribution of each esterase to ACh hydrolysis. Rather, these experiments were intended primarily as an indicator to determine if cholinesterase was sufficiently inhibited by MIX to account for MEPP, EPP, and iontophoretic potential time course prolongations.

Tenuissimus muscles from each cat were surgically removed, and carefully weighed. Sufficient 0.1 M phosphate buffer at pH8 was added to maintain a concentration of 20 mg of tissue per ml of buffer. The muscle was homogenized using a manual glass tissue homogenizer surrounded by ice to retard cholinesterase destruction.

Experimental trials simultaneously compared absorbencies of homogenate blanks which did not contain the substrate with cuvettes containing the source of the enzyme and the substrate (experimental cuvette). Each cuvette included the following:

Blank Cuvette	Experimental Cuvette
0.1 ml aliquot of muscle homogenate	0.1 ml aliquot of muscle homogenate
2.9 ml phosphate buffer	2.9 ml phosphate buffer
100 ul DTNB reagent	100 ul DTNB reagent
MIX	MIX
20 ul phosphate buffer	20 ul acetylthiocholine substrate

Both cuvettes were placed in a Beckman DU spectrophotometer and absorbence was recorded at 412 nm. Control and treatment periods each lasted 21 minutes and consisted of 22 separate absorbence recordings taken at one minute intervals. Blank cuvettes were used to correct for absorbence changes not related to acetylthiocholine hydrolysis. Enzymic activity was determined for control homogenates and homogenate containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 mM concentrations of MIX. The extinction coefficient* for the yellow anion was previously determined (Ellman et al.,1961) and the enzymatic substrate degradation rate would be related to absorbence rate by means of the following equation:

$$\text{rate (moles/l per min)} = \frac{\text{absorbence/min}}{1.36 \times 10^4}$$

Substrate hydrolysis rate calculation for muscle and other tissues included a correction for tissue concentration. Thus:

$$R = \frac{A}{1.36 \times 10^4} \times \frac{1}{(100/3120)\ C_o} = (2.30 \times 10^{-3}) \frac{A}{C_o}$$

(Ellman et al., 1961)

* The extinction coefficient also designated molar absorptivity, is a constant (E) whose value for specific units depends upon the solvent and the temperature as well as upon the wave length of light, the concentration of the colored substance and the depth of solution traversed by the light (Sandell, 1965). The extinction coefficient is related to the above variables by the following equation:

$$Ecl = \log 10^{Io/I} \quad \text{(Sandell, 1965)}$$

where c is the concentration of the colored substance, l is the depth of the solution traversed by the light, Io is the intensity of the incident light beam, and I, is the intensity of the transmitted light beam. The value of E is necessary to convert absorption rates to absolute units, i.e., (moles/l. per min) (Ellman et al.,1961).

where:

R = rate in moles of substrate hydrolyzed per min. per g of muscle;

A = change in absorbence per min.;

1.36×10^4 = extinction coefficient (E);

C_o = original concentration of tissue expressed as mg/ml [following a Lowry protein analysis of the muscle homogenate, C_o was ultimately expressed as the original concentration of tissue homogenate protein in mg/ml].

A Lowry (1951) protein assay was carried out next. This determination relies on a graded blue intensity of the alkaline copper test reagent-protein complex; the intensity was proportional to the amount of protein present. Six ml of an alkaline copper solution and 0.5 ml of diluted 1:1 Folin reagent were added to 10, 20, 50 and 100 µl samples of 1 mg/ml albumin. Thirty minutes later absorbencies were read for each sample at 500 and 750 mµ wavelengths. The known protein amounts (standards) were expressed as % absorbencies recorded in a Beckman DU spectrophotometer. The same procedure, to determine protein content, was then repeated for same amounts of muscle homogenate. Mean protein content for all muscles used were then calculated on the basis of the control albumin absorbencies in order to express substrate hydrolysis as moles of substrate hydrolyzed per minute per gram of protein.

Hydrolysis rates of all experiments for seven MIX concentrations and controls were expressed as means. A paired t-test comparing the control values and enzymatic activities in the presence of MIX were carried out. A graph was constructed with increasing MIX concentrations

represented on the abscissa versus substrate hydrolysis rate and cholinesterase inhibition on the ordinates (see Results).

Recording MEPPs In Ca^{++} Free Medium

Experimental protocol and recording circuits for these experiments were identical with those employed for the measurement of EPPs and MEPPs described earlier. Krebs solution was modified to eliminate all Ca^{++} while a high 3.74 mM Mg^{++} concentration was maintained. NaCl concentration was decreased by 0.14 mM to retain perfusate isotonicity. This modification of the perfusate resulted in the inhibition of all EPPs while MEPPs were still present. 0.025 mM, 0.1 mM and 0.5 mM concentrations of MIX were tested. MEPP amplitude and frequency data for all three concentrations were gathered and expressed as means.

Muscle Contracture

Muscles were surgically removed and one end affixed to a tissue bath holder while the other end was attached to a Harvard isometric muscle transducer. Original resting muscle tension was approximated by adjusting the in vitro preparation to the length observed in vivo. The entire preparation was immersed in an oxygenated tissue bath maintained at 37°C. Muscle contractures were recorded by ink pen deflections of a Harvard Apparatus recorder.

5.0 mM bath concentrations of caffeine, theophylline and MIX were each tested separately for their ability to induce muscle contracture. Since the contractures were irreversible only one drug could be tested in each preparation. The pertinent experiments were intended as a qualitative evaluation of contracture liability of the three

xanthines. Accordingly, muscle tension developed from resulting contractures were translated by an isometric transducer into recording pen deflections.

Indirect-Direct Muscle Twitch Recordings

Tenuissimus muscle-nerve preparations were employed for this series of experiments. Opposite ends of the muscle were attached to a tissue holder and a Harvard isometric muscle transducer. An oxygenated tissue bath set at 37°C was used. A pair of isolated silver electrodes were brought in contact with the nerve to stimulate the preparation indirectly. Silver field and tendon electrodes were used for direct muscle stimulation. A dual channel Grass S-88 stimulator supplied alternating supramaximal indirect with direct pulses. Responses were recorded on a Harvard Apparatus ink pen recorder. For both direct and indirect stimulation, threshold voltage was first determined, then quadrupled to ensure supramaximal pulses. For indirect stimuli, 16 V pulses with a duration of 0.2 msec were generally employed; each direct pulse approximated 30 volts and lasted 5 msec. Time intervals between alternating stimuli was 5 seconds.

A bath concentration of 0.5 mM MIX was tested with respect to both direct and indirect twitch heights. A 0.9 to 1.5 μg/ml bath concentration of d-Tc was also employed to partially block indirect twitch response prior to MIX administration.

The experimental protocol employed is described in detail in conjunction with the results.

Krebs Perfusates

Components of normal and modified Krebs-Henseleit Solutions employed in this study were as follows:

Components	Normal Krebs	Low Ca^{++}/High Mg^{++} Krebs	Ca^{++} Free/High Mg^{++} Krebs
KCl	4.60 mM	4.60 mM	4.60 mM
KH_2PO_4	1.15 mM	1.15 mM	1.15 mM
$CaCl_2.2H_2O$	2.46 mM	.98 mM	-
$MgSO_4.7H_2O$	1.15 mM	3.74 mM	3.74 mM
NaCl	115 mM	113.9 mM	114.88 mM
D-glucose	8.85 mM	8.85 mM	8.85 mM
$NaHCO_3$	24.1 mM	24.1 mM	24.1 mM

RESULTS

Effect of MIX on Miniature Endplate Potentials

Initial studies of MIX action at the neuromuscular junction concerned various parameters of the miniature endplate potential (MEPP). In these experiments 0.5 mM, 1.0 mM and 1.5 mM MIX concentrations were utilized.

At 0.5 mM, MIX significantly depressed MEPP amplitude from mean values of 0.61 mV to 0.55 mV while significantly elevating MEPP frequency from mean values of 0.93 MEPPs/sec. to 1.74 MEPPs/sec. (Figure 5; Table 1). Rise time and half decay time, however, were not significantly altered by this dose. Post treatment rinse resulted in the restoration of the altered parameters closer to control values (Table 1).

In addition to depressing MEPP amplitude and increasing their frequency, the two higher MIX concentrations caused significant and dose dependent prolongation of MEPP time course. For example, rise time and half decay time of MEPPs by 1.5 mM concentrations of MIX were significantly affected while the 1.0 mM concentration significantly prolonged the rise time (Table 1). Depression of MEPP amplitude was also dose dependent. Reversal of drug effects was less pronounced during the post treatment rinse period following the two higher MIX concentrations as compared with the 0.5 mM concentration. Resting membrane potentials were not significantly altered by any of the three MIX concentrations tested. Table 1 summarizes these results which are based on fourteen MEPP experiments.

Histograms were constructed in order to compare MEPP frequency and amplitude distributions during control and treatment periods for the low, 0.5 mM and high 1.5 mM concentrations of MIX. MEPP amplitude

TABLE 1

MEPP - DOSE RESPONSE STUDY

	C-I (Control)					T (Treat)					C-II (Rinse)				
	RMP mV ± S.E.	Amplitude mV ± S.E.	Frequency MEPPS/sec. ± S.E.	Rise Time msec. ± S.E.	1/2 Fall msec. ± S.E.	RMP mV ± S.E.	Amplitude mV ± S.E.	Frequency ± S.E.	Rise Time ± S.E.	1/2 Fall ± S.E.	RMP mV ± S.E.	Amplitude mV ± S.E.	Frequency ± S.E.	Rise Time ± S.E.	1/2 Fall ± S.E.
.5 mM MIX N = 4	62.5 ± 1.55	.61 ± .02	.93 ± .10	.57 ± .08	1.50 ± .15	63.0 ± 1.22	.55 ± .07 *	1.74 ± .19 *	.62 ± .11	1.69 ± .10	62.5 ± .96	.60 ± .04 ***	1.48 ± .29	.79 ± .07	1.90 ± .26
1.0 mM MIX N = 5	66.2 ± 2.67	.71 ± .08	1.31 ± .52	.87 ± .09	1.67 ± .09	65.6 ± 1.94	.57 ± .04 *	2.24 ± .60 *	1.92 ± .52 *	3.23 ± .52	67.4 ± 2.80	.69 ± .07 ***	1.09 ± .32	1.23 ± .21 **	2.28 ± .31
1.5 mM MIX N = 5	65.2 ± 2.11	.62 ± .02	.67 ± .13	.87 ± .06	1.71 ± .16	65.2 ± 2.33	.46 ± .02 *	1.33 ± .26 *	1.45 ± .17 *	3.21 ± .06 *	67.4 ± 1.91	.56 ± .05	1.74 ± .39 **	1.00 ± .02	2.17 ± .19 ** ***

*P < .05 (paired t-test) C-I vs. T.
**P < .05 (paired t-test) C-I vs. C-II.
***P < .05 (paired t-test) T vs. C-II.

All recordings were made during minutes 8-10 of each of the three 10 minute periods (CI, T, C-II).

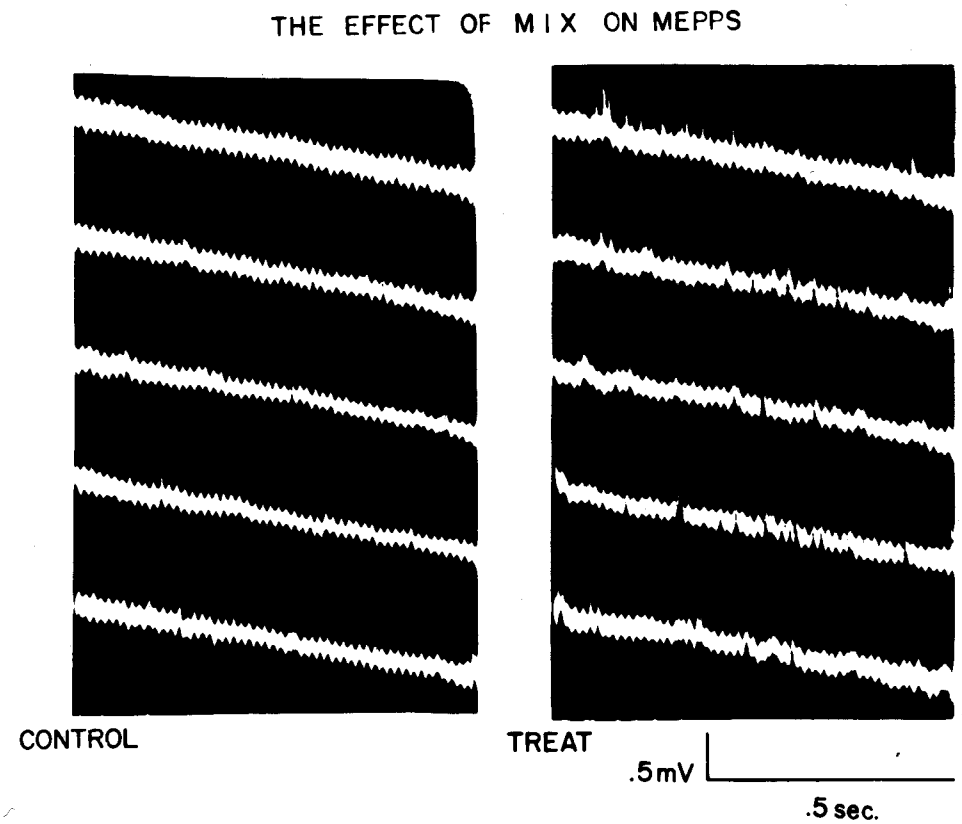


Figure 5. The above film records demonstrate the amplitude and frequency of MEPPs under control conditions (left block). Addition of 0.5 mM MIX to the perfusate results in the significant alteration of MEPP amplitude and frequency (right block). Each sweep is 1 second in duration.

distribution was shifted to the left following treatment with 0.5 mM MIX (Figure 6A). The shift was more pronounced following the treatment with a 1.5 mM concentration (Figure 6B). Altogether, the significant depression of MEPP amplitude by three concentrations of MIX tested were dose dependent (Table 1).

MEPP frequency was significantly increased by all three concentrations of MIX (fourteen experiments). However, this effect did not appear to be dose dependent (Table 1). Frequency distribution histograms characteristic of the random nature of spontaneous discharge (Fatt and Katz, 1952) were compiled. The mean frequency was shifted from 0.93 MEPPs/sec to 1.74 MEPPs/sec, a 87% increase following perfusion with 0.5 mM MIX (Figure 7A). The application of 1.5 mM MIX changed the mean frequency from a control value of 0.67 MEPPs/sec to 1.33 MEPPs/sec, a 99% increase (Figure 7B).

Since MIX exhibited dose dependent effects on MEPP amplitude, varying MIX concentrations were employed frequently in other experiments as well. However, for comparative purposes, the effects of a 0.5 mM MIX concentration were studied in all experiments. Indeed, in pilot experiments as well as in the course of this study, this concentration elicited consistent and marked effects. Furthermore, higher MIX concentrations produced results that were not reversible under the conditions of my experiments; for example, during the 10 minute post treatment rinse period following application of 0.5 mM MIX, MEPP parameters usually returned toward control values while reversal of MIX action following rinsing was less readily apparent at MIX concentrations $>$ 1.0 mM. It was felt that the use of such concentrations would preclude obtaining meaningful results and obscure the demonstration of specific MIX actions.

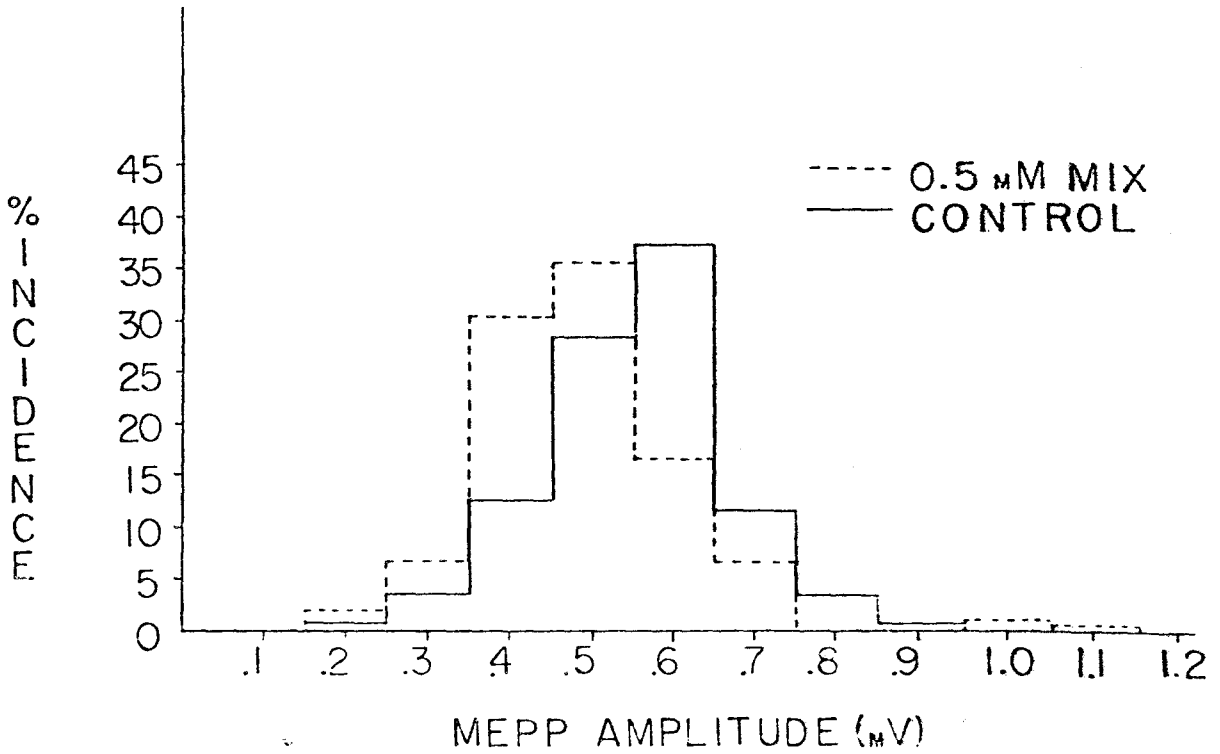


Figure 6A. MEPP amplitude histogram of pooled data from 4 experiments. 0.5 mM MIX decreased MEPP amplitude.

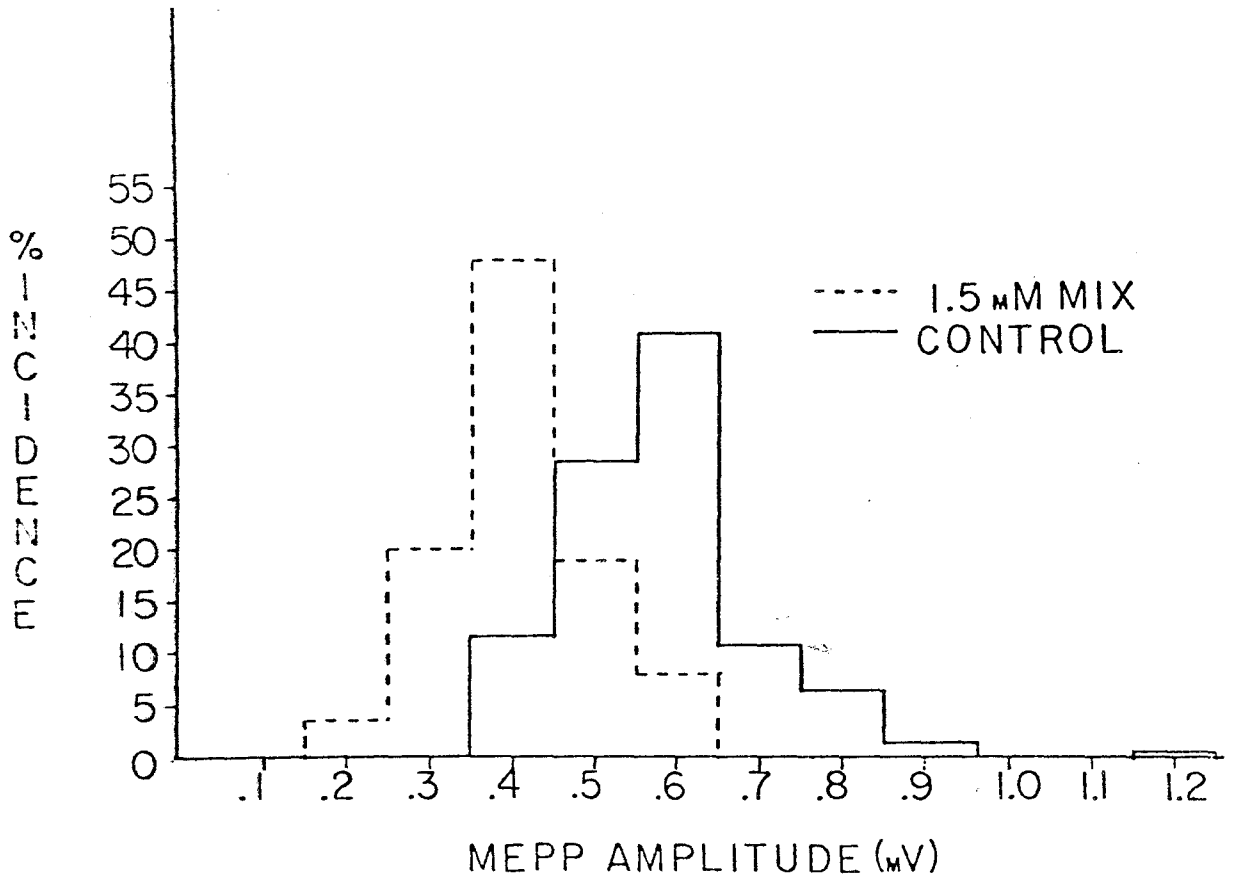


Figure 6B. MEPP amplitude histogram of pooled data from 5 experiments. 1.5 mM MIX decreased MEPP amplitude.

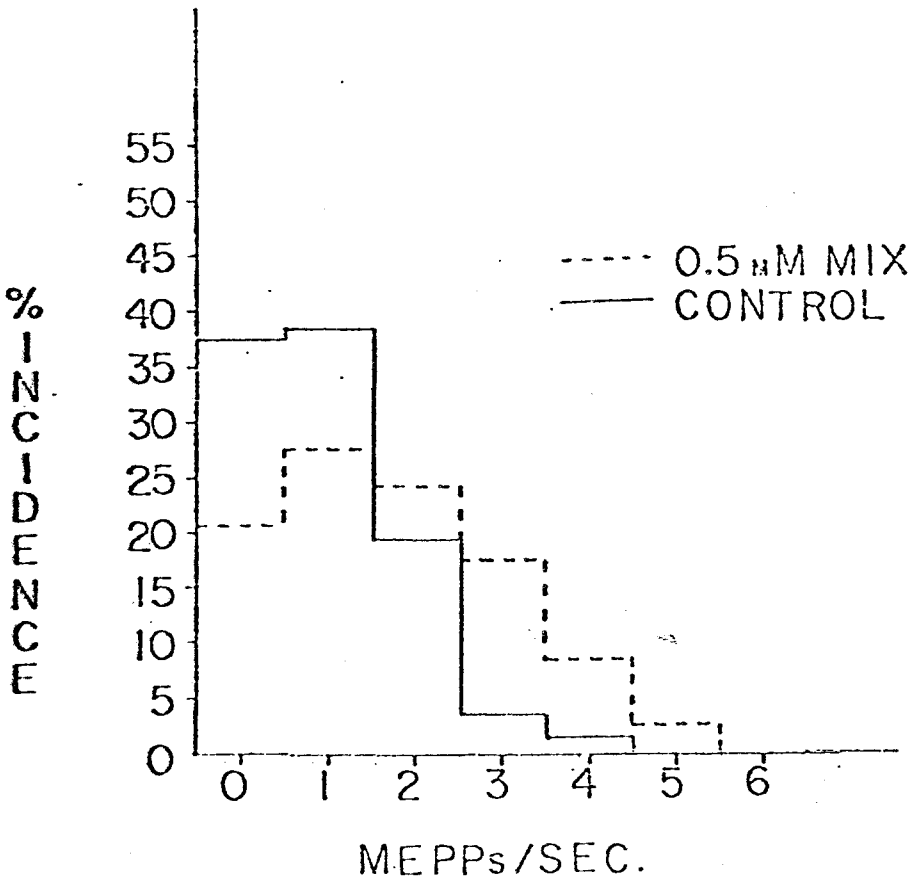


Figure 7A. MEPP frequency histogram of pooled data from 4 experiments. 0.5 mM MIX increased MEPP frequency.

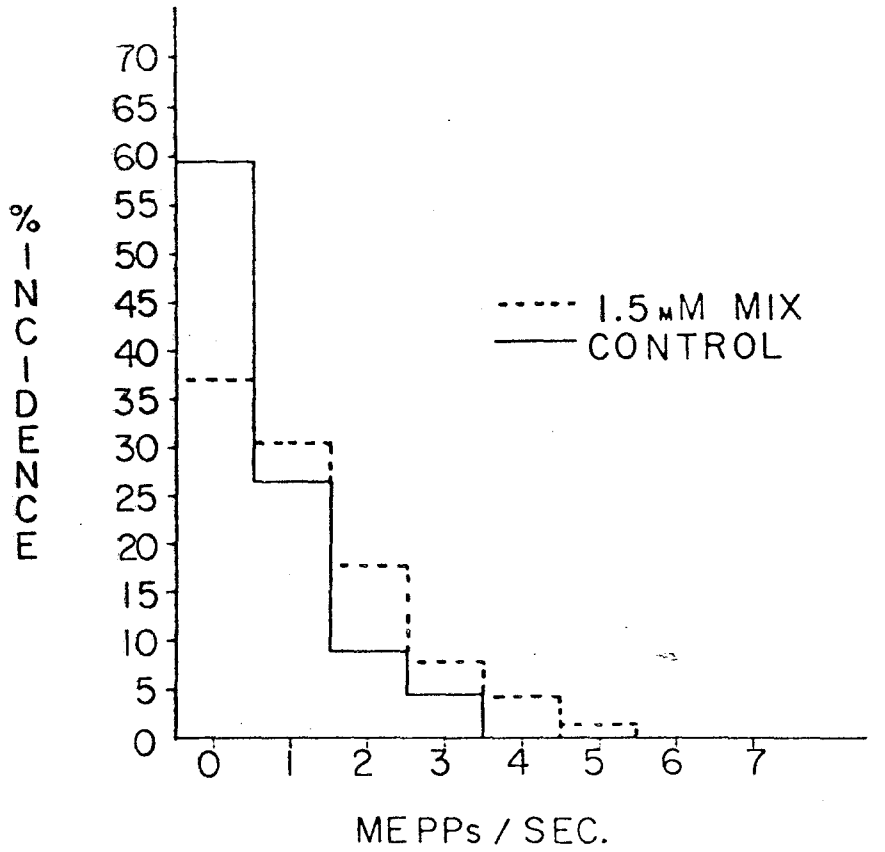


Figure 7B. MEPP frequency histogram of pooled data from 5 experiments. 1.5 mM MIX increased MEPP frequency.

Effect of MIX on Endplate Potentials

Next, MIX actions were explored with respect to evoked endplate potentials (EPPs). Muscle contraction was blocked with 2.5 μg/ml d-tubocurarine (d-Tc). 0.5 mM concentrations of MIX significantly increased EPP amplitude from mean values of 1.8 mV to 2.4 mV. The rise time and half decay time were also significantly prolonged. Mean resting membrane potentials remained unchanged throughout the duration of the experiments. EPP parameters returned to control values in the course of the ten minute rinse period. These results are summarized in Table 2; an actual recording of typical control and experimental EPPs can be seen in Figure 8.

In some experiments, 0.5 mM MIX was sufficient to increase EPP amplitudes past the threshold for muscle action potentials and to cause muscle twitch. Following a twitch, the experiment was concluded and the results not used for statistical analysis as in such cases muscle movements frequently damaged the electrode or caused a shift in its position.

In view of enhanced facilitation and occasional restoration of twitch by 0.5 mM concentrations of MIX, higher MIX concentrations were not employed. The effects of lower MIX concentrations were not studied with respect to EPPs but were evaluated with respect to other experimental paradigms.

Effects of MIX on EPP Trains and EPP Failures

The method of Elmqvist and Quastel (1965b) was employed in the determination of quantal parameters from trains of EPPs.

TABLE 2

EFFECT OF 0.5 mM METHYL ISOBUTYL XANTHINE ON ENDPLATE POTENTIALS

Treatment	N	RMP (mV ± S.E.)	EPP Amplitude (mV ± S.E.)	EPP Rise Time (msec. ± S.E.)	EPP Half Decay Time (msec. ± S.E.)
A) Control	7	71 ± 2.8	1.8 ± .18 mV	0.76 ± .03 msec.	1.3 ± .22 msec.
B) 0.5 mM MIX	7	71 ± 3.8	2.4 ± .26 mV*	0.94 ± .05 msec.*	1.8 ± .15 msec.*
C) Rinse	7	71 ± 3.9	2.3 ± .21 mV	0.74 ± .08 msec.	1.6 ± .18 msec.

*$P < 0.01$ (Paired t-Test) Treatment vs. Control

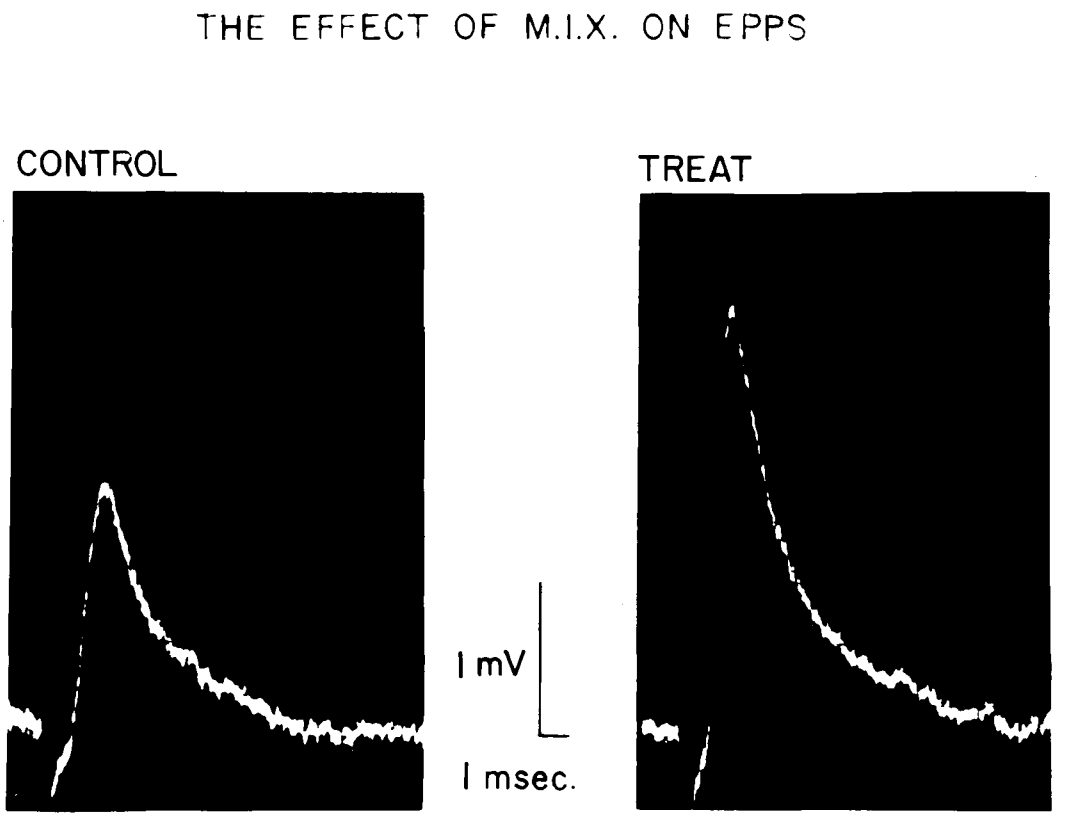


Figure 8. Effect of 0.5 mM MIX on the endplate potential. MIX significantly increased amplitude and rise time.

Trains of EPPs were obtained by indirect repetitive stimulation of the preparation at 50 Hz for 2 sec. and d-Tc was perfused to prevent muscle contraction. The primary reason for obtaining train data was to support or reject the possibility of drug related prejunctional sites of action.

MIX increased the amplitude of EPPs. The first and last EPPs of typical trains are shown in Figure 9; a 0.5 mM concentration of MIX increased the amplitudes of EPPs throughout the train. Additionally, the effect of MIX on the first EPP was evaluated and it was significantly elevated in the presence of the compound (Table 3).

A compiled summary of quantal analysis parameters obtained from seven different preparations is shown in Table 4. A 0.5 mM concentration of MIX was the only concentration used in these experiments. As can be seen quantal size (q) was significantly depressed by MIX. The mean quantal content of the head of the train, m_o (the 1st EPP), and that of the tail of the train, m_t (the last 85 EPPs), were both significantly elevated in the presence of 0.5 mM MIX. As applied to the equation m = pn*, the analysis demonstrated also that readily releasable stores (n) were significantly increased. The probability of release was unchanged.

Some of the values obtained from the quantal (variance) analysis of EPP trains prompted further tests to determine the reliability of such data for several reasons: First, MIX did not markedly increase

* m signifies quantal content;
  p signifies probability of release; and
  n signifies readily releasibly stores.

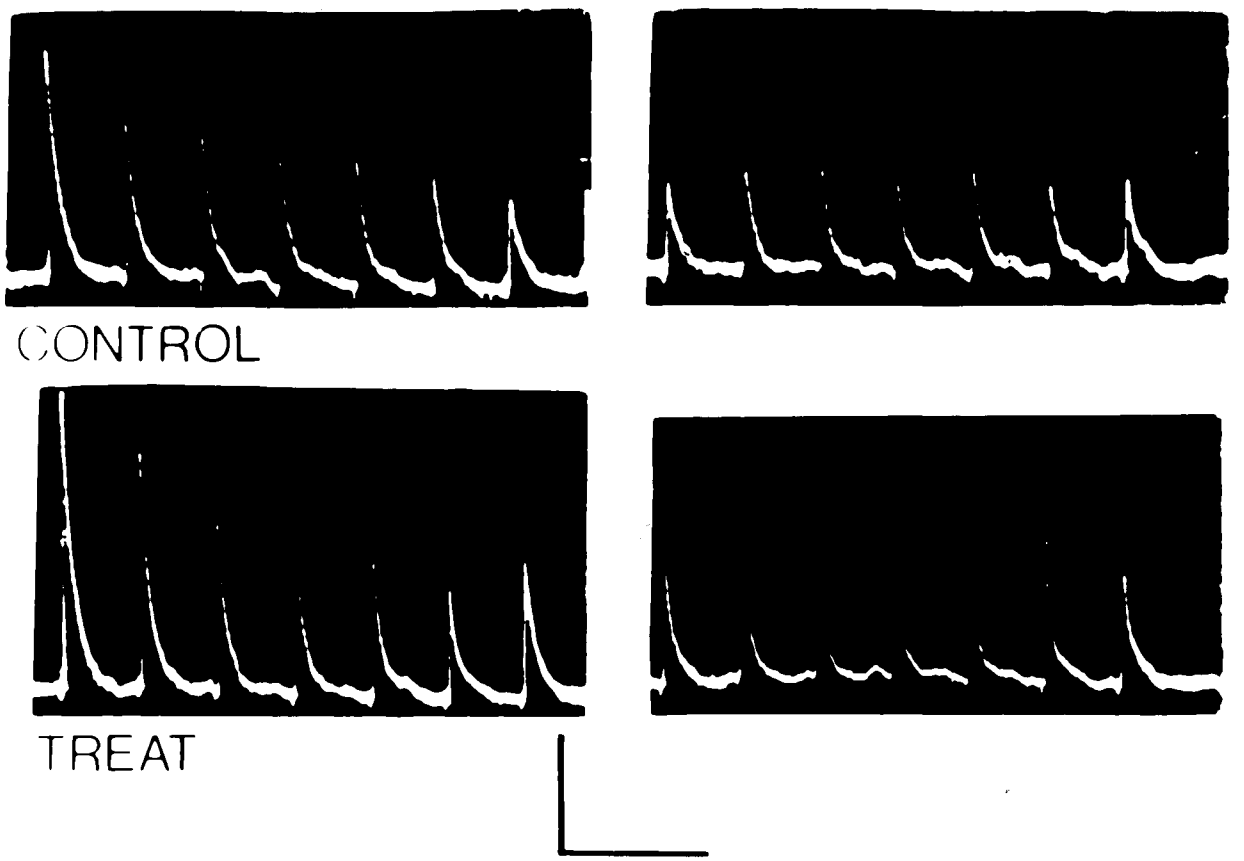


Figure 9. The two upper panels represent the first 10 and final 10 EPPs of a control train.

The two lower panels represent the first 10 and final 10 EPPs of a train during the final minute of a 0.5 mM treatment cycle.

Trains consisted of 100 impulses. The nerve was stimulated at a frequency of 50/sec. for 2 sec. These experiments were performed in the presence of 2.5 ug/ml d-Tc.

Cal: 1 mV., 60 msec.

TABLE 3

AMPLITUDE OF THE FIRST EPP OBTAINED

FROM TRAINS IN THE PRESENCE OF CURARE

Control		0.5 mM MIX Treat		Rinse	
RMP	1st EPP	RMP	1st EPP	RMP	1st EPP
- 83	2.50	- 81	2.79*	- 81	2.87
± 2.6	± 0.25	± 2.5	± 0.24	± 4.9	± 0.35
mV	mV	mV	mV	mV	mV
N = 7		N = 7		N = 4	

*p < .05 (paired t-test) control vs. treat.

The above values are expressed as means ± the standard error.

TABLE 4

QUANTAL ANALYSIS PARAMETERS

OBTAINED FROM TRAINS OF EPPS IN CURARE

CONTROL

$\bar{q}$	$\bar{mo}$	$\bar{mt}$	$\bar{p}$	$\bar{n}$
0.0175	160	67	0.21	796
± .0016	± 30	± 10	± 0.02	± 162

N = 7

0.5 mM MIX TREAT

$\bar{q}$	$\bar{mo}$	$\bar{mt}$	$\bar{p}$	$\bar{n}$
0.0140	228	83	0.21	998
± .0013	± 48	± 13	± 0.02	± 149
*	*	*		*

N = 7

RINSE

$\bar{q}$	$\bar{mo}$	$\bar{mt}$	$\bar{p}$	$\bar{n}$
0.0163	253	92	0.23	1063
± .0044	± 103	± 31	± 0.04	± 348

N = 4

---

q - quantal size
mo - quantal content of the head of the train (first EPP)
mt - quantal content of the tail of the train (all EPPs other than the 1st 15)
p - probability of release
n - readily releasable stores

*$p < .05$ (paired t-test) control versus treat.

The above values are expressed as means ± the standard error.

probability of release and this finding was inconsistent with the effects of other xanthines including caffeine and aminophylline, both of which significantly increase p (Wilson, 1973, 1974a; Shinnick, 1974). Second, the probability of release values (p = 0.22) obtained from the EPP train analysis were calculated on the assumption that quantal release was distributed according to Poisson statistics. However, in regard to quantal analysis, Johnson and Wernig (1971) stated that "Poisson predictions differed significantly from the observed quantal distributions for values of $p > 0.2$." As a result, the reliability of the quantal parameters shown in Table 4 remained questionable.

Consequently, the statistical methods applied by Johnson and Wernig (1971) were used to determine if the quantal release patterns approximated a Poisson or binomial distribution. For this analysis, independent EPP data was obtained from experiments in which a low 0.98 mM Ca^{++}/high 3.74 mM Mg^{++} modified perfusate resulted in occasional failures of EPPs to indirect stimulation. Simultaneous direct observation of both EPPs and MEPPs was required for the determination of m. Mean quantal content was obtained from the following relationship:

$$m = \frac{\text{total no. of quanta released}}{\text{number of trials}}$$

For binomial calculations the probability of release was determined by:

$$p = 1 - \sigma^2/m$$

where σ^2 is the variance of the series of trials (N). The readily releasable store (n) was obtained by the ratio m/p.

The number of failures per trial sample predicted by binomial statistics was calculated by the equation:

$$n_o = N\ (1-p)^n$$

where N is the number of trials, p is the probability of release and n is the value for the readily releasable store. For EPPs having 1, 2, 3....x* quanta the values n_1, n_2, n_3....n_x, predicted by binomial statistics could be calculated by the equation:

$$n_x = n_{x-1}\ \frac{m-p\ (x-1)}{x\ (1-p)}$$

where n_x represents the frequency of occurrence of each value of x* during a series of trials (N).

The calculation of the number of failures in the case of a Poisson distribution is given by:

$$n_o = Ne^{-m}$$

while the frequency of occurrence of each value of x during N trials is calculated by the equation:

$$n_x = n_{x-1}\ (m/x)$$

Resulting distributions of quanta from 15 experiments calculated from the above equations are presented in Table 5. Table 5 clearly shows that for both control and treatment conditions 6 of 7 of the observed quantal distributions were closer to the values of the expected binomial

* The x subscript represents any integral 1 or greater to designate those EPPs containing that number of quanta.

TABLE 5

QUANTAL DISTRIBUTION

	Control						
	n_0	n_1	n_2	n_3	n_4	n_5	n_6
Observed	662	1087	1278	515	163	32	4
Binomial	666	1268	1139	533	164	39	4
Poisson	824	1178	899	329	219	83	27
	B	P	B	B	B	B	B

	MIX Treatment						
	n_0	n_1	n_2	n_3	n_4	n_5	n_6
Observed	382	814	1307	785	358	130	13
Binomial	341	908	1155	893	455	99	28
Poisson	608	948	873	621	372	191	109
	B	B	B	B	P	B	B

Table 5. The above values indicate that observed quantal distributions agree more closely with calculated binomial distributions than with Poisson distributions. These data also represent pooled values from 15 EPP failure experiments. n_0 designates all EPPs having 0 quanta (failures), n_1 all EPPs having 1 quanta, etc. P or B under each column signifies if the observed values agree more closely with Poisson or binomial. See text for calculations.

distribution. In accordance with the analysis by Johnson and Wernig (1971) a chi square test applied to the quantal distributions of individual experiments revealed that 9 of 14 experiments revealed goodness of fit to a binomial distribution while all of the expected Poisson distributions were significantly different ($P < .05$) when compared to the observed distributions. Thus, observed distributions could not be characterized as being Poisson in nature. As a result, the variance analysis of EPP trains have provided data which could no longer be considered appropriate since the calculation of such data was based on the assumption that quantal phenomena could be described by Poisson distribution. Nevertheless, these (Table 4) parameters were retained here for the purpose of comparison and discussion. The quantal parameters calculated on the basis of binomial distribution are summarized in Table 6. Corresponding parameters from EPP train analysis were also re-included (from Table 4) to emphasize the inconsistencies between the two sets of data. Thus, for several MIX concentrations tested, probability of release is significantly increased and readily releasable stores are insignificantly diminished as determined by the binomial analysis (Table 6). In contrast, the probability of release parameter is unchanged while readily releasable stores are significantly increased by 0.5 mM MIX as determined by the inappropriate variance analysis.

Despite major inconsistencies between the two approaches to quantal analysis the inappropriate EPP train data does reveal some trends consistent with other independent experiments. For example, the drug related decrease in quantal size correlates well with the significant

TABLE 6

EPP FAILURE QUANTAL ANALYSIS DATA

Number of Experiments	Dose	$\bar{m}$	$\bar{p}$	$\bar{n}$	$\overline{\text{Failures}}$/Impulses
4	Control	1.94	.39	6.48	32/250
4	0.5 mM MIX	2.65*	.61*	4.66	8/250
3	Control	1.19	.30	4.23	65/250
3	0.1 mM MIX	2.27*	.60*	3.79	14/250
4	Control	1.83	.32	17.7	33/250
4	0.05 mM MIX	2.37*	.51	5.00	10/250
4	Control	1.39	.35	4.72	52/250
4	0.006 mM MIX	1.18	.34	4.28	68/250

EPP TRAIN QUANTAL ANALYSIS DATA FROM TABLE 4

Number of Experiments	Dose	$\bar{mt}$	$\bar{p}$	$\bar{n}$	$\overline{\text{Failures}}$/Impulses
7	Control	67.01	.21	796	None
7	0.5 mM MIX	83.83	.22	998	None
4	Rinse	92.87	.23	1063	None

*$p < .05$ (paired t-test) control versus MIX treated.

depression of MEPP amplitude described earlier. Quantal content was also seen to increase significantly in both (train and failure) analyses following MIX concentrations ranging from 0.01-0.5 mM and in accordance with direct observations.

The evidence, up to this point, does not appear to suggest a facilitatory postjunctional site or sites of drug action. Such effects of MIX as enhancement of MEPP frequency, increased EPP amplitude as juxtaposed with depressed MEPP amplitude, and increases in quantal content and probability of release suggest a predominantly prejunctional facilitatory site or sites of MIX action. Several additional experimental paradigms were employed to conclusively determine whether or not MIX possessed postjunctional actions in the cat.

Effects of MIX on Depolarization Due to Acetylcholine Perfusion

Perfusion of the preparation with medium containing 10-20 μg/ml acetylcholine (ACh) chloride was carried out until two consecutive uniform endplate depolarizations were obtained at 10 minute intervals. A third ACh depolarization was obtained near the end of a 0.5 mM MIX perfusion period (treatment response). Ten minutes later, a final ACh depolarization in the absence of MIX served as a post-treat control.

The results, expressed as means of thirteen such experiments, are presented in Table 7. The mean depolarization due to combined ACh-MIX treatment was not significantly different from that obtained in controls. Perhaps most striking are the identical means of the second control depolarization (dep. 2) and the depolarization obtained in the presence of MIX (dep. 3), both having mean values of 9.9 mV.

TABLE 7

ACETYLCHOLINE PERFUSION DEPOLARIZATION EXPERIMENTS

Depolarization 1 ACh		Depolarization 2 ACh		Depolarization 3 ACh + MIX 0.5 mM		Depolarization 4 ACh	
RMP	dep. 1	RMP	dep. 2	RMP	dep. 3	RMP	dep. 4
- 74	- 9.8	- 74	- 9.9	- 75	- 9.9	- 75	- 10.8
± 2.4	± 1.6	± 2.2	± 1.1	± 2.5	± 1.6	± 4.8	± 2.3
mV	mV	mV	mV	mV	mV	mV	mV
N = 13		N = 13		N = 13		N = 4	

In these trials an ACh concentration of 10-20 μg/ml was employed.

None of the above treatment values differed significantly from control.

These data are expressed as means ± standard error.

Depolarization of individual muscle cells in response to ACh perfusion was demonstrated in the frog (Ochs and Mukheyee, 1959; Katz and Miledi, 1961) and in the rat (Thesleff, 1955). Katz and Miledi (1961) stated that such depolarizations originated at junctional endplates since receptor free regions revealed diminished sensitivity even to increased ACh concentrations. These authors also reported a mean ACh depolarization of 9 mV (from 11 fibers) in the presence of ACh, 10^{-5}M and eserine, 10^{-5}M (Katz and Miledi, 1961). The magnitude of these depolarizations correlates well with those reported in MIX experiments. Since d-Tc and eserine modified the depolarization response to ACh (Thesleff, 1955; Katz and Miledi, 1961) such experiments might serve as a pharmacological indicator of postjunctional receptor sensitivity. Such was the rationale in selecting ACh depolarization trials in the presence and absence of MIX. However, the negative results reported here with MIX, combined with the rather prolonged ACh depolarization and recovery times (often taking minutes), prompted the decision to use a more precise technique, namely, microiontophoresis.

Iontophoretic Application of ACh

In brief, iontophoretic ACh potentials were evoked by ejection of ACh, via current pulses, from a micropipette positioned extracellularly in the endplate region. Experiments were initiated only when successive ACh potential amplitudes were consistent and uniform. A 0.5 mM concentration of MIX was the only concentration employed in this series of experiments and characteristic responses obtained in a typical experiment are shown in Figure 10. Mean amplitudes, time course determinations and resting membrane potential values from seven iontophoresis

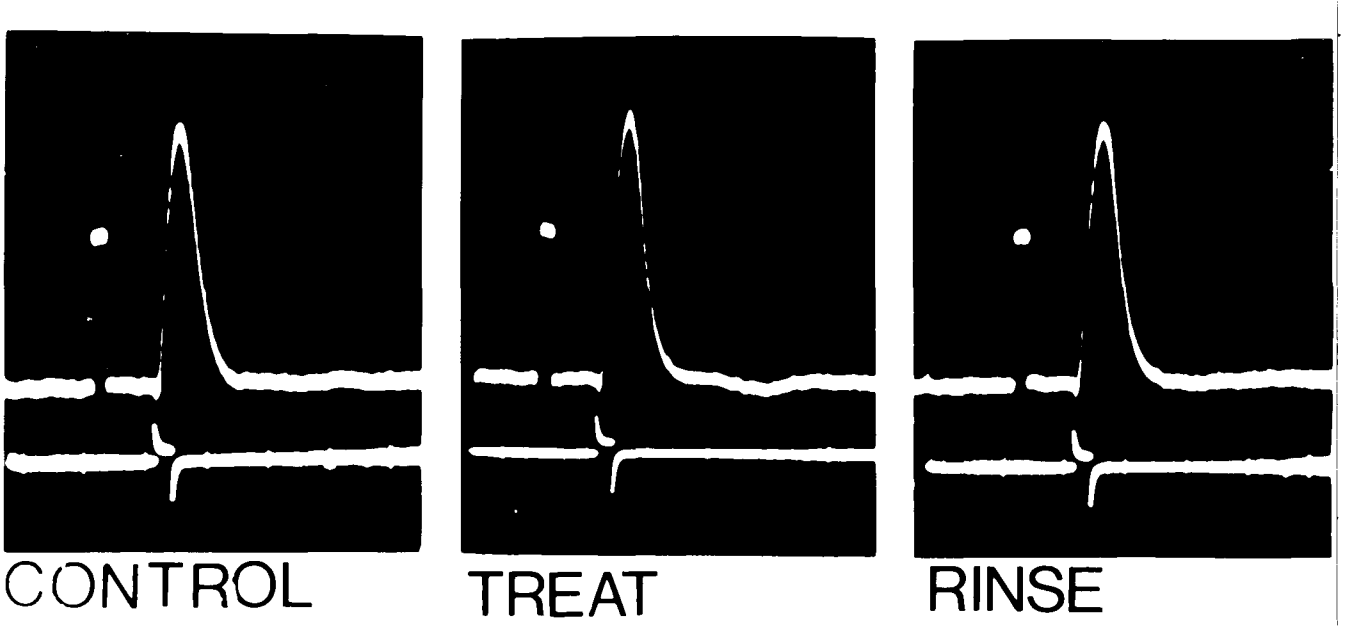


Figure 10.

Upper Traces
Potentials evoked by iontophoretic application of ACh 0.5 mM MIX application demonstrates no significant alteration of the potential. Calibration pulse 5 mV, 20 msec.

Lower Traces
ACh ejection pulses 20 msec. duration and 1.9×10^{-8} AMPS.

experiments were compiled (Table 8). Only the rise time was significantly prolonged by 0.5 mM MIX. However, the mean resting membrane potential (RMP) and the iontophoretic potential amplitude recorded during drug washout was significantly elevated when compared to controls. The significant hyperpolarization may account for the accompanied significant elevation of post-treatment wash amplitudes. If the values obtained during MIX treatment rather than those obtained during the washout period are considered for comparison with the control values, then the results of iontophoresis experiments suggest that at a 0.5 mM concentration, MIX does not induce postjunctional facilitation of the ACh potential.

Input Membrane Resistance

Recording of input membrane resistance from a single cell required the insertion of two microelectrodes that served for evoking the electrotonic hyperpolarizing potential via a current pulse and for recording this potential. This electrode arrangement was responsible for the low RMPs observed in these experiments. Figure 11 illustrates an example of the hyperpolarizing electrotonic potentials obtained from a single cell; in these experiments R was computed on the assumption that Ohm's Law was obeyed for small hyperpolarizations (Hodgkin and Rushton,1946). When comparable current pulses were employed for the control and drug treatment periods, the associated electrotonic potentials (Figure 11) were not significantly altered. In the typical experiment MIX was unable to alter input membrane resistance. Furthermore, the time constants, Tm, for each electrotonic potential were determined on the assumption that the potential change was an error function of time

TABLE 8

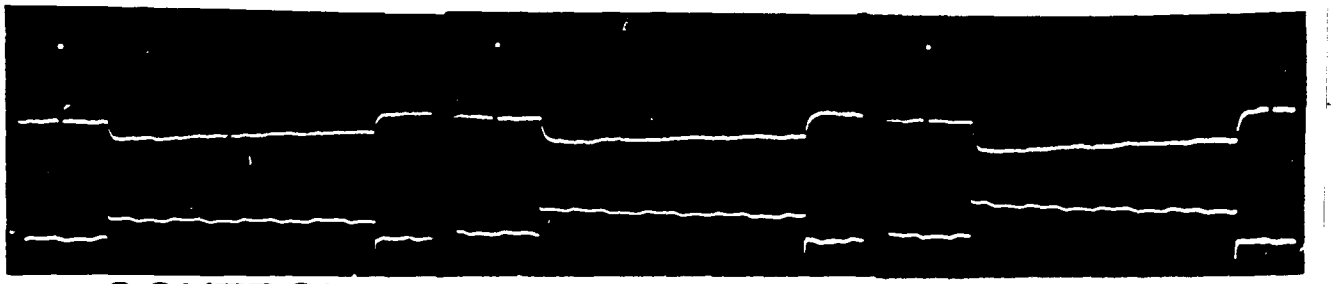
ACh IONTOPHORESIS EXPERIMENTS

	RMP mV	Amplitude mV	Rise Time msec.	1/2 Decay Time msec.	N
Control	-59.0 ± 3.04	6.10 ± .61	127.89 ± 28.27	123.20 ± 21.48	7
0.5 mM MIX	-60.7 ± 2.18	7.02 ± 1.32	146.37** ± 27.36	146.56 ± 25.91	7
Rinse	-65.6* ± 1.51	7.41* ± 1.02	147.99 ± 36.42	146.73 ± 28.44	7

*$p < .05$ (paired t-test) control versus post treat control rinse.

**$p < .05$ (paired t-test) control versus 0.5 mM MIX treat.

THE EFFECT OF MIX ON ELECTRONIC POTENTIALS



CONTROL

TREAT

UPPER TRACE CAL. 10 mV. 1 MSEC.

LOWER TRACES; INCREASING CURRENT PULSES; 70 MSEC.

Figure 11. Electrotonic potentials in the absence and presence of MIX. There was no effect.

(Hodgkin and Rushton, 1946). There was no significant change in their value following a 0.5 mM concentration of MIX. Table 9 summarizes the results of nine experiments involving two concentrations of MIX. At 0.5 and 2.0 mM concentrations, MIX did not alter input membrane resistance or time constant.

In experiments with 0.5 mM MIX a gradual depolarization was observed. This decrease in RMP during the post-treatment interval was significant. In this case, the inability of the selected cells to maintain a constant RMP over prolonged (> 20 min.) periods most likely resulted from the penetration of relatively small cells by two large tip diameter electrodes. In contrast, application of 2.0 mM MIX consistently resulted in a pronounced sudden and significant drop in resting membrane potential which was partially reversible upon rinsing. This phenomenon suggests a depolarizing action of MIX at high (2.0 mM) concentrations.

Figure 12 represents current voltage plots of control and treatment data. The slopes of these lines did not differ significantly in accordance with the pooled data of Table 9.

The negative amplitude findings from ACh iontophoresis and ACh perfusion experiments enhance support for the idea that significant postjunctional MIX actions are absent. Nevertheless, increases in EPP amplitude together with prolonged EPP, MEPP and iontophoretic potential time course measurements may suggest that MIX exhibits anticholinesterase properties. In contrast, MIX induced depression of MEPP amplitude and its inability to augment ACh potential amplitudes is not consistent with this conjecture. In view of possible conflicting data, a cholinesterase assay was carried out with increasing MIX concentrations.

TABLE 9

INPUT MEMBRANE RESISTANCE

Control			0.5 mM MIX Treat			Rinse		
$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$	$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$	$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$
49	3.30	3.25×10^5	48	3.30	3.03×10^5	45	3.34	2.72×10^5
$\pm$	$\pm$		$\pm$	$\pm$		$\pm$	$\pm$	
4.6	0.28		4.8	0.24		4.0	0.50	
mV	msec.	Ω	mV	msec.	Ω	mV ***	msec.	Ω
N = 6			N = 6			N = 6		

Control			2.0 mM MIX Treat			Rinse		
$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$	$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$	$\overline{RMP}$	$\overline{Tm}$	$\overline{R}$
49	-	2.98×10^5	30	-	2.56×10^5	37	-	2.55×10^5
$\pm$			$\pm$			$\pm$		
5.2			3.2			4.5		
mV		Ω	mV		Ω	mV ** ***		Ω
N = 3			N = 3			N = 3		

*p < .05 (paired t-test) control vs. treat.
**p < .05 (paired t-test) control vs. post treat control.
***p < .05 (paired t-test) treat vs. post treat control.

None of the above resistance changes are significant.

Resting membrane potential (RMP), time course ($\overline{Tm}$) and input resistance (R) data are reported for 0.5 mM and 2.0 mM MIX concentrations. Every trial with 2.0 mM MIX resulted in cell depolarization. Subsequent experiments employed MIX concentrations < 2.0 mM.

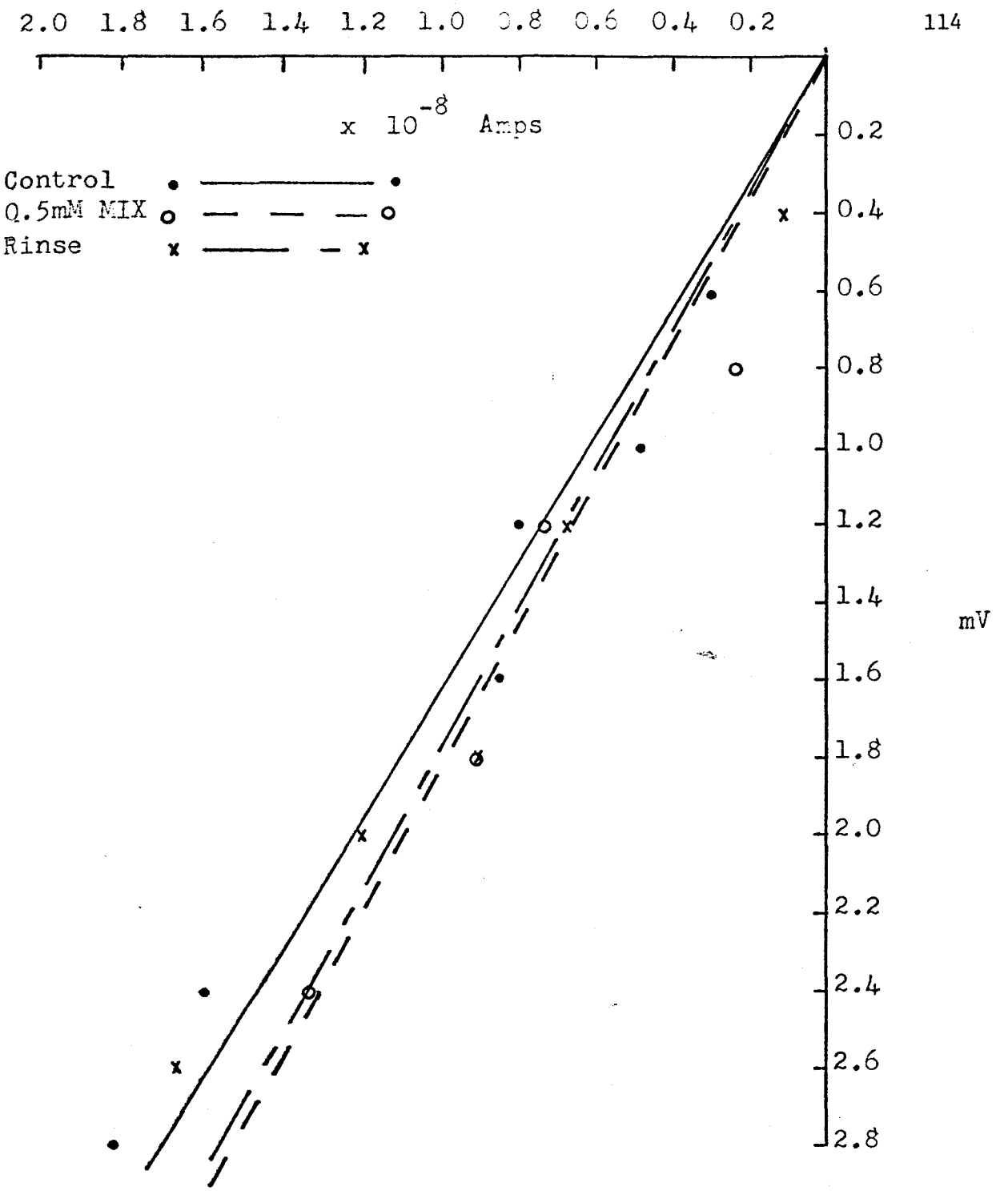


Figure 12. Effect of 0.5 mM MIX on current voltage plots. Best fit lines were constructed by linear regression from data obtained from a typical experiment. Statistical comparison of the slopes was made by covariance analysis. Slopes were not significantly different.

Anticholinesterase Properties of MIX

The effect of several MIX concentrations on cholinesterase activity of cat muscle homogenates was determined by using the colorimetric method of Ellman et al. (1961). In addition, a protein assay was undertaken in order to determine the amount of protein contained in the homogenates. Thus, the degree of cholinesterase inhibition could be correlated with the rate in moles of substrate hydrolyzed/min./g. of protein. This assay included seven MIX concentrations ranging from 0.5 mM to 5.0 mM. The substrate (acetylthiocholine) hydrolysis rate versus MIX doses are shown in Table 10. Each value represents the mean rate $\pm$ the standard error for eight experiments. At 0.5 mM MIX, 5.40×10^{-4} moles of acetylthiocholine were hydrolyzed per minute per gram of protein representing approximately a 22% inhibition of cholinesterase. A 50% inhibition of the enzyme occurred at a MIX concentration of 3.0 mM. Altogether, muscle cholinesterase inhibition ranged from approximately 22% to 55% with 0.5 to 5.0 mM MIX concentrations.

MEPPs in Ca^{++} Free Medium

The striking reversal of EPP failure rate by MIX reported previously (see Table 6) prompted another investigation of MEPPs in which a Ca^{++} free/high (3.74 mM) Mg^{++} perfusate was employed. This ionic modification totally abolishes EPPs whereas MEPPs remained present (Fatt and Katz, 1952). This observation suggests that an independent intracellular Ca^{++} requirement exists for spontaneous, in contrast to evoked transmitter release which is dependent mainly on extracellularly Ca^{++} (Alnaes and Rahamimoff, 1975). Consequently, Ca^{++} free MEPP

TABLE 10

ANTIACETYLCHOLINESTERASE ACTIVITY OF MIX

Control	.5 mM MIX	1.0 mM MIX	1.5 mM MIX	2.0 mM MIX	2.5 mM MIX	3.0 mM MIX	5.0 mM MIX
7.01	5.40	4.94	4.64	4.28	3.76	3.52	3.18
± .77	± .67 *	± .50 *	± .51 *	± .52 *	± .42 *	± .40 *	± .67 *

*$p < .0005$ (control vs. treat).

The above values are expressed as activity x 10^{-4} moles of substrate hydrolyzed/min./gram of protein.

experiments were designed to supply indirect evidence for a possible MIX role in the activation of nerve terminal Ca^{++}.

The Ca^{++} free experiments also served to accept or reject the idea that a nerve terminal depolarization caused by MIX may be the underlying cause for elevated MEPP frequency. This cause-effect relationship is based on the following rationale. Asynchronous ACh release resulting from nerve terminal depolarization is dependent on an adequate extracellular Ca^{++} concentration (Liley, 1956b). Hence, if MIX did cause nerve terminal depolarization, the increased MEPP frequency effect would be absent or diminished in Ca^{++} free medium. Such was not the case.

The MEPP data obtained in these experiments are essentially identical with those obtained earlier in non-modified medium. As shown in Table 11, a 0.5 mM concentration of MIX caused a significant MEPP amplitude depression while significantly elevating the MEPP frequency (Figure 13). Lower concentrations of MIX (0.1 mM and 0.025 mM) revealed no significant alterations of amplitude but MEPP frequency remained significantly elevated. In all twelve experiments resting membrane potentials were not altered.

Muscle Contracture Trials

This series of experiments was carried out to indirectly show whether or not MIX is capable of activating muscle Ca^{++} stores. Indeed, compounds which evoke muscle contracture responses are thought to mobilize muscle cell Ca^{++} (Bianchi, 1961).

Exposure of isolated frog sartorious muscle preparations to 5.0 mM concentrations of theophylline or caffeine resulted in irreversible muscle contractures. In contrast to other xanthines, 5.0 mM MIX

TABLE 11

MEPP DOSE RESPONSE STUDY IN Ca^{++} FREE MEDIUM

	C-I (Control)			T (Treat)			C-II (Rinse)		
	RMP (mV) ± S.E.	Amplitude (mV) ± S.E.	Frequency MEPPS/sec. ± S.E.	RMP (mV) ± S.E.	Amplitude (mV) ± S.E.	Frequency MEPPS/sec. ± S.E.	RMP (mV) ± S.E.	Amplitude (mV) ± S.E.	Frequency MEPPS/sec. ± S.E.
.5 mM MIX N = 4	69.0 ± 5.7	.50 ± .05	2.80 ± .36	69.8 ± 4.3	.42 ± .04 ***	4.38 ± .78 *	69.8 ± 3.6	.46 ± .05	3.73 ± .36 **
.1 mM MIX N = 4	70.3 ± 5.12	.48 ± .04	1.26 ± .64	70.0 ± 5.23	.45 ± .02	2.16 ± 1.01 *	69.5 ± 5.39	.45 ± .02	1.81 ± 1.26
.025 mM MIX N = 4	67.3 ± 2.29	.57 ± .06	1.19 ± .15	65.3 ± 3.20	.53 ± .05	1.98 ± .43 *	62.8 ± 4.27	.54 ± .06	1.91 ± .46

*p < .05 (paired t-test) C-I vs. T
**p < .025 (paired t-test) C-I vs. C-II
***p < .005 (paired t-test) C-I vs. T

Perfusion medium contained no calcium and an elevated magnesium concentration (3.74 mM M_g^{++}). Isotonicity was retained by elevating the [NaCl] by .98 mM. greater than the low Ca^{+}/high Mg^{++} medium.

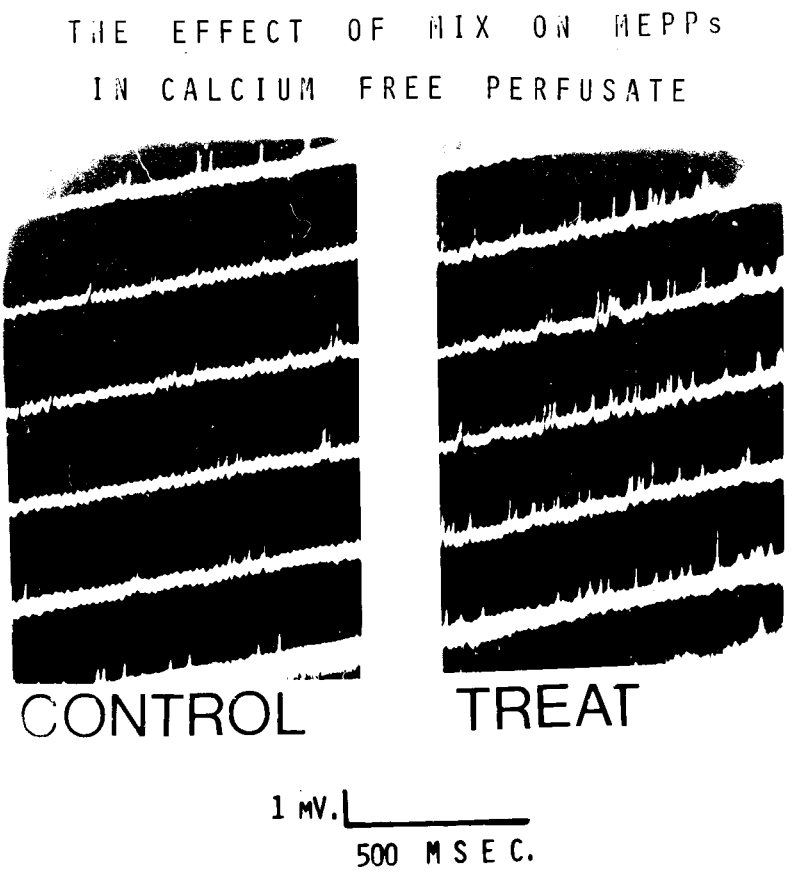


Figure 13. Effect of 0.5 mM MIX on MEPPs in calcium free perfusate.

demonstrated no alteration of muscle tension or contracture. The maximal tensions obtained by each preparation were translated by a force transducer into recording pen deflections inscribed on moving chart record paper. Such inscriptions illustrating the results of a typical experiment are presented in Figure 14. The results obtained with each drug were computed from 8 to 11 trials per drug and are presented in Table 12. Irreversible contracture tensions were induced in all the preparations employed by 5.0 mM concentrations of caffeine or theophylline; in sharp contrast, no tension increase resulted from the application of 5.0 mM concentrations of MIX. These data suggest that MIX lacks the muscle Ca^{++} activation properties characteristic of the other xanthines tested.

Experiments with Alternating Direct-Indirect Muscle Twitch Responses

In order to demonstrate MIX action on muscle twitches, in vitro muscle nerve preparations were stimulated in an alternating direct indirect manner. In evaluating the data obtained in these experiments the maximum twitch response to direct stimulation was assigned a value of 100%. Thus, maximum twitch height of the response to direct stimulation served as a control with which other twitches were compared.

0.5 mM concentrations of MIX resulted in a significant increase in twitch height response to indirect stimulation from mean values of 39.7% to 64.0%. This junctional facilitation is consistent with the microelectrode data already obtained.

Twich response to direct stimulation was also facilitated; the effect of 0.5 mM MIX, while small (8%) was significant.

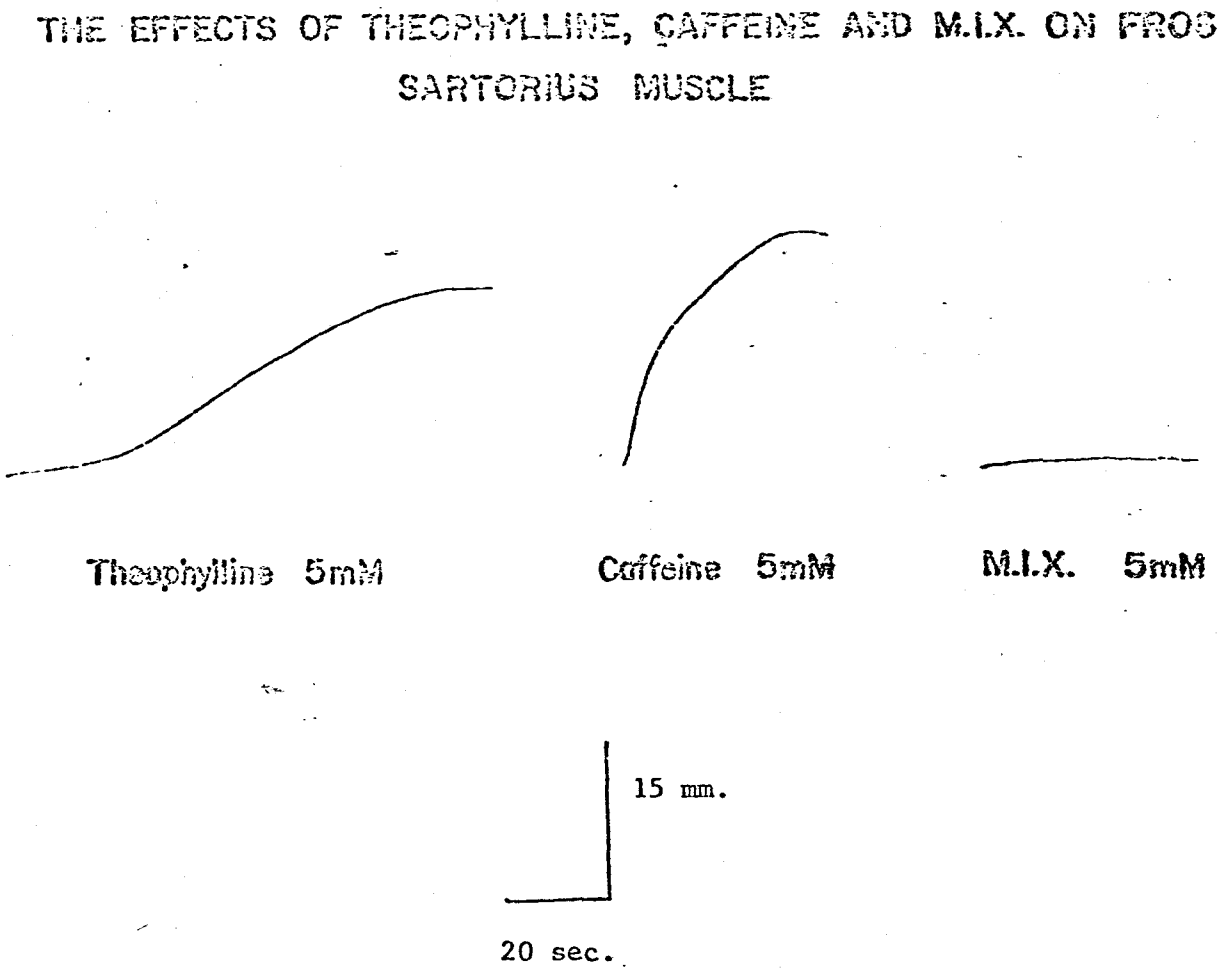


Figure 14. Identical concentrations of three methylxanthines were tested for their ability to induce irreversible muscle contracture. Theophylline and caffeine demonstrated this effect while MIX at no time caused contracture. The ordinate calibration represents distance traversed by a recording pen on chart paper in response to tension developed by isometrically contracted muscles.

TABLE 12

ISOMETRIC MUSCLE CONTRACTURE TENSIONS

EVOKED BY 5.0 mM CONCENTRATIONS OF XANTHINES

Mean Max. Tension 5 mM Theophylline	Mean Max. Tension 5 mM Caffeine	Mean Max. Tension 5 mM MIX
17.5 ± 6.1 mm	27.0 ± 4.9 mm	.500 ± .38 mm
*	*	
N = 8	N = 11	N = 8

*p < .05 paired t-test (MIX vs. caffeine or theophylline).

The above values are expressed as the means ± standard error of the distance in mm traversed by a recording pen. The tension developed by muscle contracture was translated by an isometric force transducer into recording pen movement.

In ensuing experiments, the effect of MIX on d-Tc induced neuromyal block was evaluated. Preliminary trials showed that 0.5 mM concentrations of MIX did not reverse d-Tc induced depression of twitch response to indirect stimulation when the depression was greater than 50%. Consequently, the concentration of d-Tc was gradually increased to cause twitch depression ranging from 30% to 50%. In four experiments, d-Tc caused a mean depression of twitch response to direct stimulation by 15% and a mean depression of twitch response to indirect stimulation by 32%; only the depression of the indirect responses was significant. Subsequent application of 0.5 mM concentrations of MIX caused a significant increase of both indirectly and directly obtained twitch responses over the d-Tc depressed values.

Recordings from two typical experiments are shown in Figure 15. The data are summarized in Table 13.

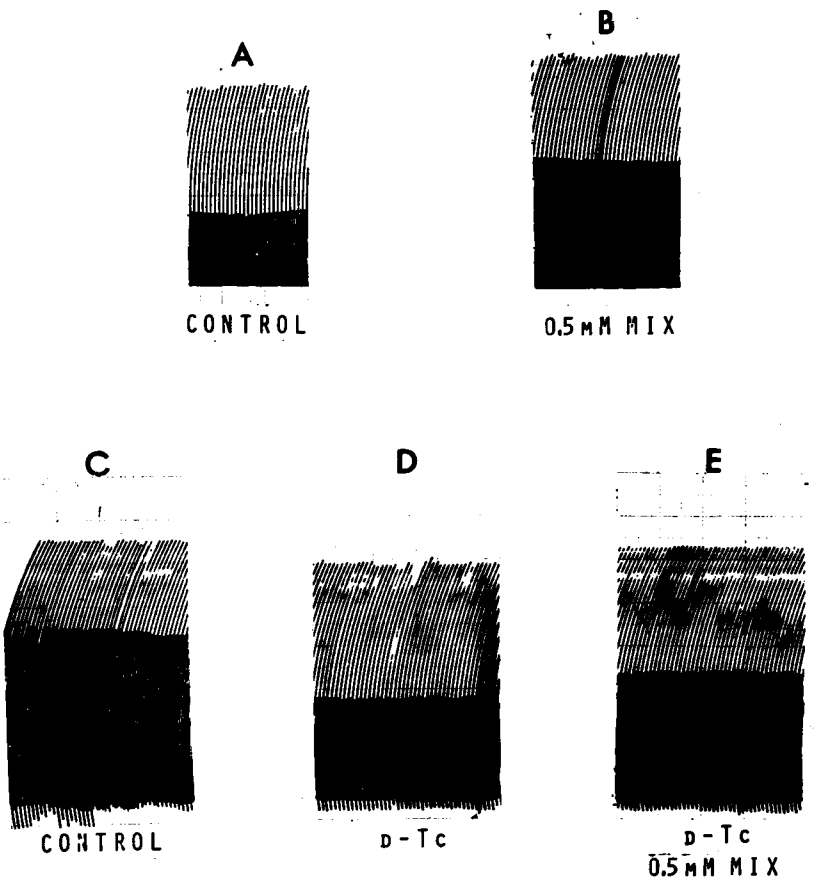


Figure 15. Traces A and B represent control and 0.5 mM MIX treatment responses to an isolated nerve muscle preparation being stimulated directly (lighter trace) and indirectly (darker trace) in an alternating sequence. Traces C, D and E are similar recordings to those in A and B. However, sufficient d-Tc was added to impair neuromuscular transmission by 33%. 0.5 mM MIX demonstrated a slight anticurare action.

TABLE 13

D_{MAX}	$\overline{I_{MAX}}$	[MIX]	$\overline{D_{MIX}}$	$\overline{I_{MIX}}$
100%	39.75 ± 9.20%	.5 mM	108.75 ± 4.15% #	64.00 ± 11.54% ##

#p < .05 (paired t-test) I_{MAX} versus I_{dTc}.
##p < .05 (paired t-test) I_{MAX} versus I_{MIX}.

D_{MAX}	$\overline{I_{MAX}}$	[$\overline{dTc}$]	$\overline{D_{dTc}}$	$\overline{I_{dTc}}$	[MIX]	$\overline{D_{MIX}}$	$\overline{I_{MIX}}$
100%	62.25 ± 6.3%	1.05 ug/ml	85.00 ± 8.13%	42.50 ± 9.13% *	.5 mM	90.75 ± 6.66% **	55.00 ± 12.06% ***

*p < .05 (paired t-test) I_{MAX} versus I_{dTc}.
**p < .05 (paired t-test) D_{dTc} versus D_{MIX}.
***p < .05 (paired t-test) I_{dTc} versus I_{MIX}.

D_{MAX} - The maximum response to applied direct stimuli. This response was assigned a value of 100% and functioned as a control with which other direct responses were compared.

I_{MAX} - The maximum response to applied indirect stimuli expressed as a % of the D_{MAX} value. The I_{MAX} value functioned as a control with which other indirect responses were compared.

[MIX] - Bath concentration of MIX.

[dTc] - Mean bath concentration of dTc.

D_{MIX} - .5 mM drug treatment response after direct stimulus plateau is attained. D_{MIX} is expressed as a % of the D_{MAX} value.

I_{MIX} - .5 mM drug treatment response after indirect stimulus plateau is attained. I_{MIX} is expressed as a % of the D_{MAX} value.

D_{dTc} - Curare treatment response after direct stimulus plateau is attained. D_{dTc} is expressed as a % of the D_{MAX} value.

I_{dTc} - Curare treatment response after indirect stimulus plateau is attained. I_{dTc} is expressed as a % of the D_{MAX} value.

NOTE: Actual twitch response amplitudes were compared for statistical significance prior to their expression as percentages.

DISCUSSION

Collective consideration of all MIX experiments carried out at the neuromuscular junction indicate primarily a prejunctional site of action. Supportive findings include the following: significantly increased MEPP frequency with no increased amplitude; increases of EPP amplitude, quantal content and probability of release. The absence of drug related alterations of ACh perfusion depolarization potentials, iontophoretic potentials, input membrane resistance and time constant determinations all indicate a deficiency of postjunctional involvement by MIX. This brief summary is intended to put the pharmacological actions of MIX into perspective. The remainder of this discussion, however, will focus upon more detailed explanations for the mechanisms and sites of MIX actions as they relate to the above findings.

Spontaneous Release and Ca^{++}

In the cat tenuissimus nerve-muscle preparation MIX concentrations ranging from 0.5 mM to 1.5 mM altered significantly three MEPP parameters; MEPP time course was prolonged, amplitude was depressed while frequency was elevated. Several MIX concentrations of less than 0.5 mM also caused an elevation of MEPP frequency while not affecting amplitude. These dose related effects seemed to imply that the prejunctional sites responsible for spontaneous ACh release were more sensitive to MIX actions than were those sites associated with the alteration of other MEPP parameters (i.e., postjunctional membrane and MEPP amplitude).

Elevation of MEPP frequency is a well documented property of methylxanthines and has been described for caffeine in rat diaphragm

(Elmqvist and Feldman, 1965; Hofmann, 1969) and in frog sartorius muscle (Mambrini and Benoit, 1963). In a similar fashion MEPP frequency was enhanced by theophylline in rat diaphragm (Goldberg and Singer, 1969) and in cat tenuissimus muscle (Shinnick and Jacobs, 1971, 1972; Shinnick, 1974)*.

MEPP frequency alternations have been attributed to shifts of nerve terminal membrane potential by an agent. MIX, however, does not appear to elevate MEPP frequency by terminal depolarization. Evidence supporting the stability of nerve terminal potential in the presence of MIX is presented later in this discussion.

A complete explanation of the mechanism responsible for methylxanthine induced elevation of MEPP frequency has not been provided by any one study. In the case of theophylline several investigators have attributed enhanced MEPP frequency to prejunctional unbinding of Ca^{++} (Elmqvist and Feldman, 1965) and to a postjunctional increase in Ca^{++} efflux (Bianchi, 1961) which suggested an elevation of extracellular Ca^{++} level (Mambrini and Benoit, 1963).

MEPP amplitude depression by a given agent may occur through an action on the prejunctional nerve terminal but is more commonly related to a depression of postjunctional response (Hubbard et al. 1969a). In contrast to aminophylline which increased MEPP amplitude (Shinnick, 1974), MIX consistently depressed MEPP amplitude at higher concentrations (0.5 mM to 1.5 mM), but not at concentrations lower than 0.5 mM.

* "Theophylline" used by Shinnick et al. was in fact aminophylline and will be hereafter referred to as such. This designation is appropriate as Wilson (1973) demonstrated several differences between the actions of aminophylline and theophylline at the neuromuscular junction.

Results of muscle twitch experiments revealed that the possibility of MIX exhibiting enhanced curare like actions on postjunctional receptors is unlikely. Other data tend to suggest that MEPP amplitude depression may be prejunctional. For example, several MIX concentrations did not alter ACh potential amplitude, ACh perfusion depolarization, input membrane resistance or time constant. These findings suggest that MIX possesses a facilitatory site of action causing an increase in the frequency of spontaneous release and an inhibitory site of action causing the depression of MEPP amplitude (i.e., q size). The absence of postjunctional effects supports the concept that both sites may be located within the prejunctional nerve terminal or membrane.

Several mechanisms that may underlie the prejunctional depression of MEPP amplitude have been discussed in the literature. Elmqvist and Quastel (1965b) demonstrated that hemicholinium progressively diminished quantum size following repetitive nerve stimulation. This phenomenon was ascribed to transmitter depletion coincidental with hemicholinium-related blockade of choline uptake and depression of ACh synthesis. In contrast to hemicholinium, MIX decreased quantal size, i.e., it attenuated MEPP size in the absence of stimulation. Consequently, a hemicholinium like action could not be assigned to MIX since hemicholinium significantly diminishes q size recorded from non-depleted resting junctions.

Similar to the MEPP alterations caused by MIX, 0.06 mM vinblastine elevated MEPP frequency by 300% and diminished MEPP amplitude from 0.75 mV to 0.45 mV in frog sartorius muscle (Turkanis, 1973). No mechanism was found: a postjunctional receptor blockade or a reduction in

the amount of ACh per quanta were given as possible explanations.

A mechanism related to Ca^{++} constitutes the most plausible explanation of the MIX induced reduction of quantal size. Fatt and Katz (1952) first reported that a fourfold increase in extracellular Ca^{++} concentration produced a small diminution of the frog sartorius miniature potentials. Mambrini and Benoit (1963) observed a 40% depression of amplitude with elevated Ca^{++} concentration and inconsistent alteration of MEPP amplitude, by changing Ca^{++} levels from 2.0 mM to 5.0 mM or from 2.0 mM to 0.1 mM. A similar effect was also reported for rat diaphragm (Hubbard et al., 1968b). MEPPs of reduced amplitude were recorded in frog sartorius immersed in an isotonic medium in which Na ions were replaced by Ca ions (Katz and Miledi, 1969). Recent investigations by Kriebel and Gross (1974) and Kriebel et al. (1976) showed that a two to eight fold increase in Ca^{++} concentration reversibly increased MEPP frequency and also decreased, by up to 15%, the MEPP amplitude in mouse diaphragm preparations.

Further evidence suggesting a prejunctional site of MIX action was advanced by an investigation of Ruthenium red, an inhibitor of Ca^{++} uptake by mitochondria (Alnaes and Rahamimoff, 1975). This compound increased spontaneous transmitter release but decreased quantal size at frog motor nerve terminals. The effect of Ruthenium red on MEPP parameters was attributed to an inhibition of Ca^{++} transport into nerve terminal mitochondria resulting in higher concentrations of residual calcium. The similarity of actions of Ruthenium red and MIX on MEPPs suggests that the mechanism of action of both compounds may be similar. An alternative explanation may implicate a release or leak of intracellular

stores of Ca^{++} transport by nerve terminal mitochondria. In either case the common effect would be attributed to increased intraneuronal free Ca^{++} levels.

On the basis of extensive investigation of many tissue types Rasmussen and Tenenhouse (1968) implicated a possible functional link between cyclic AMP and Ca^{++} in the release of transmitter from motor nerve terminals. This proposal was later supported by the observed increase in MEPP frequency and decrease in MEPP amplitude by 4.0 mM dibutyryl cyclic AMP (Goldberg and Singer, 1969) and especially since Ca^{++}, dibutyryl cAMP, MIX and Ruthenium red all demonstrated a similar combination of actions on MEPPs.

MIX and Contracture

Considerable evidence for methylxanthine related Ca^{++} mobilization is consistent with the results of the present studies which suggest that the depression of MEPP amplitude and enhancement of MEPP frequency by MIX may be due to a Ca^{++} dependent mechanism. Altogether, MIX may cause Ca^{++} liberation leading to an alteration of MEPP parameters in terms of a prejunctional site of action.

Several controversial items, however, require further clarification:

A 5.0 mM MIX concentration does not evoke muscle contractures similar to those observed with equal concentrations of caffeine or theophylline. Caffeine induced irreversible muscle contractures (Axelsson and Thesleff, 1958) were considered to result from an efflux of intracellular Ca^{++} or freeing of bound Ca^{++} stores (Bianchi and Shanes, 1959; Bianchi, 1961; Herz and Weber, 1965). Such contractures

were not mediated by changes in resting potential or in the ionic permeability of the muscle membrane (Axelsson and Thesleff, 1958) and were independent of extracellular Ca^{++} levels. In contrast to methylxanthine contractures, those induced by K^{+} were characterized by reversible membrane depolarization.

Caffeine was reported to exert its effect on the external cell membrane; intracellular administration of caffeine did not result in a contracture. Bianchi (1961) theorized that caffeine reduced Ca^{++} binding sites at the muscle membrane or sarcoplasmic reticulum, hence causing increased Ca^{++} fluxes.

Lüttgau and Oetliker (1968) argued that Ca^{++} was released by the action of caffeine on T-tubules. However, Howell (1969) indicated that glycerol induced destruction of the T-tubules had no effect on caffeine contractures. He subsequently concluded that caffeine acts directly upon the sarcoplasmic reticulum membranes to induce Ca^{++} release.

More recently, Bianchi (1975) described methylxanthine contracture in detail. As he explained, this tension increase resulted from a leak of Ca^{++} from sarcoplasmic reticulum terminal sacs. During the contracture, Ca^{++} leak was greater than the rate at which Ca^{++} would be restored to the sacs via reticulum binding and transport, resulting in persistent contracture. The characteristic irreversibility requires further explanation. Bianchi (1975) postulated that caffeine in concentrations of 5.0 mM or greater also acts as an uncoupler of the sarcoplasmic reticulum energy related Ca^{++} transport. Thus while ATP continues to be utilized by sarcoplasmic reticulum via myosin ATP ase for the uptake of Ca^{++}, the latter cannot be stored. The subsequent

depletion of phosphates results in the characteristic rigor which cannot be reversed by caffeine removal (Nauss and Davies, 1966).

No convincing evidence exists for methylxanthine saturation of specific binding sites. Consequently, the precise sequence of methylxanthine induced interactions promoting calcium release are as yet unknown. However, Bianchi (1962) and Isaacson and Sandow (1967) suggested that caffeine exerts its pharmacological actions by interfering with hydrophobic bonds or lipoproteins present in the membrane. Another difficulty with respect to our understanding of the contracture mechanism concerns the differences between the potencies and rates of tension development with various xanthines. For example, while all contractures are qualitatively similar, caffeine is most potent followed by theobromine or theophylline (Axelsson and Thesleff, 1958). As the actions of caffeine or skeletal muscle were related to the degree of Ca^{++} leak developed at the sarcoplasmic reticulum, the difference in contracture among various methylxanthines might be related to the degree of Ca^{++} leak produced by each at the reticulum. In the case of MIX, the absence of related muscle contracture appears inconsistent with the myotropic actions of other methylxanthines. Yet the prejunctional actions of caffeine, theophylline and MIX appear to be similar. For a contracture activating methylxanthine such as caffeine, a 5.0 mM or greater concentration is required for contracture (Bianchi, 1975) while a 1.0 mM concentration is sufficient to evoke significant junctional effects (Wilson, 1973). However, an even greater concentration differential to distinguish between prejunctional and contractural actions may exist in the case of MIX. The insolubility of MIX at concentrations

greater than 5.0 mM prevents the testing of this hypothesis. It cannot be assumed that MIX totally is devoid of myotropic actions since MIX enhances the twitch of both directly and indirectly stimulated preparations. However, the MIX facilitation of twitch response to muscle stimulated directly may be due, at least in part, to an indirect component (i.e., nerve terminal stimulation). A 5.0 mM concentration of MIX may cause only a slow leak of Ca^{++} from the muscle sarcoplasmic reticulum. This would account for the potentiation of muscle twitchs similar to those seen with other methylxanthines at low ($\bar{}$ 1.0 mM) concentrations as well as the absence of contracture.

In another investigation utilizing frog muscle Weber and Herz (1968) demonstrated that under normal resting conditions, Ca^{++} flux was linked to ATP hydrolysis and that caffeine acts to partially uncouple this metabolic process. Such an action would then account for the Ca^{++} release associated with contracture. Although this mechanism is probably not pertinent for MIX, there remains an interesting possibility that Ca^{++} related muscle contractures by methylxanthines are closely related to metabolic processes involving ATP and ATPase. On this basis it may be speculated that, in contrast to caffeine, MIX stabilizes the Ca^{++}/ATP coupling to prevent excess release from the sarcoplasmic reticulum. Consequently, the absence of MIX contracture might be ascribed to its metabolic actions on the ATP-ATPase system in limiting Ca^{++} flux. This explanation stands in contrast to an alternative hypothesis according to which methylxanthines might directly affect the affinity or number of membrane Ca^{++} binding sites. Rasmussen and Tennhouse (1968) have also indicated that ATP and, to a lesser extent

cyclic AMP can chelate Ca^{++}. No evidence currently exists which would implicate MIX involvement in such a mechanism.

A number of MIX actions were mimicked by SQ 20009 [1-ethyl-4-(isopropyl-indenehydrazino)-1H-pyrazolo-(3, 4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl], a cyclic nucleotide phosphodiesterase inhibitor (Jacobs and McNiece, 1976). SQ 20009 caused a facilitation of nerve terminal release as evidenced by twitch, MEPP and EPP data. Passive membrane properties and membrane sensitivity to ACh were not altered. Furthermore, at the concentration of 5.0 mM SQ 20009 was incapable of inducing contracture of the frog sartorius muscle. However, increases in the Ca^{++} concentration of the perfusate from 0.7 to 1.1 mM augmented SQ 20009 actions by increasing EPP rate of rise significantly. As in the case of MIX, SQ 20009 may affect Ca^{++} release similarly. Both compounds demonstrated Ca^{++} like effects prejunctionally but failed to increase Ca^{++} flux at the sites responsible for muscle contracture. Indeed, the metabolic links between prejunctional phosphodiesterase inhibition, elevated cAMP levels and Ca^{++} mobilization constitute a probable explanation of SQ 20009 as well as the MIX mechanism of action.

MEPP and EPP Time Course

EPP and MEPP rise times and half decay times were prolonged at a MIX dose of 0.5 mM or greater. This prolongation is likewise evident in frog muscle in the presence of caffeine (Mambrini and Benoit, 1963) and in cat tenuissimus muscle following aminophylline perfusion (Shinnick, 1974).

Such a prolongation might be explained as being due to an increase in the time constant of the membrane. However, no significant alteration

of this parameter obtained from electrotonic potentials was observed in the presence of 0.5 mM MIX. Another possibility might include nerve terminal depolarization. This proposal is unlikely as the possibility of nerve terminal depolarization is reduced in low or Ca^{++}/free/high Mg^{++} medium; yet, MEPP frequency and amplitude were affected similarly by MIX in both normal and in Ca^{++} free/high Mg^{++} Krebs perfusate. Longer time course might also result from drug related prolongation of transmitter release although no evidence implicating MIX in this phenomenon is available.

Time course prolongation might be linked to anti-ChE action of MIX. At the concentration of 0.5 mM, MIX caused a 22% inhibition of ChE; even at 5.0 mM, MIX induced depolarization of the endplate yet only a 55% inhibition of ChE. Usually cholinesterase inhibition as great as 60% to 90% is required before twitch potentiation is observed (Barnes and Duff, 1953; Meer and Meeter, 1956). MIX findings, however, do not rule out the possibility of MEPP or EPP prolongation via ChE inhibition since the degree of ChE inhibition has not been simultaneously related to the parameters of MEPPs or EPPs by past investigators. Thus, it is conceivable that even a limited inhibition of ChE by MIX may contribute partially to prolonged EPP and MEPP time course.

EPP Amplitude

Amplitudes of single EPPs as well as of EPP trains evoked by repetitive stimuli were consistently and significantly enhanced in the presence of 0.5 mM MIX. Comparable increases in EPP amplitude were caused by theophylline and aminophylline (Wilson, 1974a; Shinnick, 1974). Mambrini and Benoit (1963) did not observe consistent increases in quantal

content in the presence of caffeine, and hence suggested that the increases were the result of a post-junctional alteration of receptor sensitivity. In regard to theophylline, however, Goldberg and Singer (1969) presented evidence against the possibility of enhanced receptor sensitivity; a 1.9 mM concentration of theophylline elevated MEPP frequency significantly without altering MEPP amplitude. An enhanced receptor sensitivity should be accompanied by an increase in MEPP as well as EPP amplitude. Shinnick (1974) and Shinnick et al. (1976) demonstrated evidence for both pre and post-junctional aminophylline actions on cat muscle. In view of theophylline reversal of d-Tc depression of MEPPs and increased EPP amplitude, aminophylline actions could not be attributed solely to an elevated quantal content.

Wilson (1973) also observed significantly increased EPP amplitudes in the presence of caffeine. He related the effect to a Ca^{++} mechanism since both caffeine and Ca^{++} similarly elevated quantal content, probability of release and mobilization rate. Hofmann (1969) attributed caffeine related EPP facilitation to increases in the mobilization rate and in the size of the releasable store. Caffeine induced enhancement of spontaneous release was attributed to an increase in the probability of release (Hubbard et al.,1968a). Goldberg and Singer (1969) were convinced that caffeine facilitated nerve terminal ACh release via cyclic AMP thus causing increased EPP amplitude. Despite diverse opinions as to the precise site of caffeine or theophylline actions the consensus among investigators favors the presence of a link between elevated quantal content and increased EPP size.

Some investigators have concluded that compounds which mobilize

calcium are characterized by their ability to increase probability of release and quantal content whereas readily releasable stores were not affected (Hubbard et al.,1968b; Wilson, 1974b). On the other hand, compounds which elevate cAMP were shown to increase readily releasable stores and mobilization rate but not the probability of release (Wilson, 1974a; Hofmann, 1969). Still other investigators favor a link between cyclic AMP and Ca^{++} in junctional facilitation (Breckenridge et al.,1967; Goldberg and Singer, 1969; Rasmussen, 1970). This relationship will be explored in detail further in this discussion.

In the case of MIX, a lack of post-junctional findings suggest that EPP facilitation is primarily due to the elevation of quantal content. This possibility is further supported by the absence of a MIX induced increase in MEPP amplitude since any alteration of the post-junctional receptor would be accompanied by comparable changes in MEPP and EPP amplitude (Goldberg and Singer, 1969).

Quantal Analysis

Mean quantal parameters investigated in the course of low Ca^{++}/ high Mg^{++} EPP failure experiments included quantal content (m) probability of release (p) and readily releasable stores (n). Other parameters including mobilization (d_m) and quantal size (q) were obtained from analysis of EPP trains. However, cat tenuissimus junctional release was shown to be binomial rather than Poisson in nature. Thus, quantal (train) analysis was no applicable. Furthermore, Wilson (1973) showed that d-Tc increased the probability of release values; this phenomenon may have contributed, at least in part, to a shift in the nature of release from Poisson to binomial.

Various methods for determining quantal analysis parameters for several methylxanthines have been reported; the results are summarized in Table 14. As probable interactions of methylxanthines with Ca^{++} and/or cAMP have been postulated, the effects of the latter two agents have also been included in Table 14.

Dibutyryl cAMP increased readily releasable stores but had negligible effect on probability of release (Wilson, 1974a). Consequently, the action of dibutyryl cAMP was characterized primarily as implicating cAMP involvement in the enhanced synthesis of ACh. Theophylline actions appear to involve primarily cAMP since its actions mimic those of dibutyryl cAMP (Wilson, 1974a).

The effect of Ca^{++} on the prejunctional nerve terminal was also studied. In contrast to dibutyryl cAMP, Ca^{++} (2.0-5.0 mM) caused a significant increase in the quantal content and probability of release but did not affect readily releasable stores (Wilson, 1973). These findings are identical to those reported for MIX which strengthens the argument that prejunctional facilitation by MIX is primarily attributable to a Ca^{++} mechanism. Similarly, Hubbard et al. (1968b) stated that the primary effect of high Ca^{++} concentration was to increase the probability of release while having no significant effect on the size of the releasable store. The data included in Table 14 suggest that aminophylline and caffeine facilitated neuromyal transmission via an effect upon Ca^{++} and its action on ACh release (Wilson, 1973; Shinnick, 1974).

Interpretation of quantal analysis results obtained by different investigations requires caution. Discrepancies among investigators

TABLE 14

A COMPARISON OF QUANTAL ANALYSIS DATA

Compound	Dose	Prep/Block	$\bar{m}$	$\bar{p}$	$\bar{n}$	Reference
Caffeine	1.0 mM	Rat Diaphragm d-Tc	I	I	NC	Wilson, 1973
Theophylline	1.0 mM	Rat Diaphragm Cut Fiber	I	NC	I	Wilson, 1974a
Aminophylline	1.0 mM	Rat Diaphragm Cut Fiber	I	I	NC	Wilson, 1974a
Aminophylline	.9 mM	Cat Tenuissimus Cut Fiber	I	I	NC	Shinnick, 1974
Aminophylline	.9 mM	Cat Tenuissimus d-Tc	NC	NC	NC	Shinnick, 1974
Dibutyryl cAMP	1.0 mM	Rat Diaphragm Cut Fiber	I	NC	I	Wilson, 1974a
Ca^{++}	5.0 mM	Rat Diaphragm Cut Fiber	I	I	NC	Wilson, 1973
MIX	0.5 mM	Cat Tenuissimus Low Ca^{++}/High Mg^{++}	I	I	NC	From This Dissertation

I Significantly increased over control values.

NC No change when compared to control values.

are common and may be due largely to differences in experimental conditions. For example, in mammalian muscle, cut fiber preparations mobilization was significantly increased by 1.0 mM aminophylline as reported by Wilson (1974a), and unchanged by 0.9 mM aminophylline as reported by Shinnick (1974). Furthermore, Wilson (1973) and Goldberg and Singer (1969) suggested that elevated cAMP levels may result in an enhanced synthesis of ACh, which is consistent with the finding of an increase in available stores following theophylline perfusions (Wilson, 1973).

Some of the procedural variations among investigators which may account for the resulting discrepancies include the following: Wilson stimulated cut fiber rat diaphragm preparations at 150/sec while Shinnick stimulated cut fiber cat tenuissimus preparations at 200/sec; furthermore, when d-Tc was employed for immobilizing the muscle, no significant changes between control and aminophylline treated preparations were observed with the exception of an increase in quantal size (Shinnick, 1974). A number of variables including animal species, muscle cell type, rate of stimulation, method of analysis, concentration of the drug, rate of perfusion, temperature, perfusate modification, condition of the preparation, etc., must be carefully considered when comparing the pertinent studies.

In analysis of EPP trains designed to investigate MIX actions, d-Tc was used for the immobilization of the muscle. However, Hubbard et al. (1969b) indicated that d-Tc increases probability of release in rat diaphragm muscle. Perhaps d-Tc also contributed to the noticeably higher p values ($p = 0.21$) in train experiments; this may have been a contributing factor to the departure of tansmitter release from Poisson characteristics.

The presence of high (> .20) p (probability of release) values are not compatible with the Poissonian nature of release. Experiments performed in low Ca^{++}/high Mg^{++} solutions not only eliminated the possibility of d-Tc elevated p values but confirmed that cat tenuissimus transmitter release was in fact binomial. With the influence of d-Tc eliminated, a p value of 0.39 in low Ca^{++} experiments was significantly increased to a p value of 0.61 by 0.5 mM MIX. EPP train experiments, on the other hand, demonstrated no change in p when comparing control (p = 0.21) to treatment (p = 0.22) values.

Postjunctional Evaluation

In view of the prolongation of MEPP and EPP time courses and depressions of MEPP amplitude by MIX, a postjunctional evaluation of MIX actions was carried out. ACh-induced depolarization of the isolated tenuissimus preparations was not augmented by 0.5 mM MIX concentrations and no drug related increase in ACh potential obtained by iontophoretic application of ACh was noted. Furthermore, MIX had no effects on resistance or on the time constant of the membrane. However, a 0.5 mM concentration of MIX significantly prolonged iontophoretic potential rise time. This observation is consistent with comparable prolongations of EPP and MEPP rise times and may suggest some postjunctional action of the compound. The prolonged time course (rise time) might be attributable to sustained neurotransmitter release or to the previously discussed anticholinesterase properties of MIX. However, the data obtained in vitro demonstrated only a 22% inhibition of cholinesterase by a 0.5 mM concentration of MIX. This level of inhibition would appear inadequate to induce significant effects. Time course

prolongations of junctional potentials have been reported for caffeine (Mambrini and Benoit, 1963) and for aminophylline (Shinnick, 1974). Evidence necessary to explain time course prolongation by MIX is presently not available.

Ca^{++} Modified Perfusates

The necessity of Ca^{++} for the evoked transmitter release and the competition of Mg^{++} with Ca^{++} at the release triggering site are well documented (Del Castillo and Engbaek, 1954; Del Castillo and Katz, 1954a; Jenkinson, 1957; Dodge and Rahamimoff, 1967; Hubbard et al., 1968a; Cooke et al. 1973). Decreased Ca^{++} concentration of the perfusate depressed the quantal content; these ionic concentrations could be adjusted to result either in a number of EPP failures or in the suppression of individual EPPs in response to supramaximal stimuli. Difficulties concerning the induction of failures were encountered in this investigation. One to two hours perfusion with 0.98 mM Ca^{++}/ 3.74 mM Mg^{++} modified Krebs was needed with some preparations prior to the appearance of failures. Yet in other preparations the same perfusate caused 100% failure rate after shorter perfusion periods. An even lower Ca^{++}/higher Mg^{++} Krebs perfusate was not employed since its use in prior trials increased the number of preparations exhibiting 100% failure rates; such preparations could not be used for analysis. Accordingly, a standard 0.98 mM Ca^{++}/3.74 mM Mg^{++} perfusate was employed for all trials.

The EPP failures method is highly reliable for the calculation of the quantal content (Katz, 1966) provided that the release conforms to Poisson characteristics. In view of the binomial nature of release

in the case of MIX experiments the calculation of $\bar{m}$ was obtained by means of the direct method ($m = \frac{\overline{EPP\ amplitude}}{\overline{MEPP\ amplitude}}$). EPPs and MEPPs could be visualized simultaneously during EPP failure experiments. MIX induced a significant increase in the value of $\bar{m}$ and this trend was consistent with the results of the EPP train analysis in the presence of d-Tc. Furthermore, experiments involving the failure method demonstrated that MIX was effective at concentrations as low as 0.05 mM.

MIX induced reversal of failure rate appears to be dependent on increased availability of Ca^{++}. Prior experiments have supplied additional indirect evidence implicating that MIX is involved in the mobilization of Ca^{++}. MIX actions thus observed in the course of failure experiments are consistent with a Ca^{++} involvement for a number of reasons:

1. Addition of either Ca^{++} or MIX to preparations exhibiting low Ca^{++}/high Mg^{++} induced failures results in a significant decrease or elimination of failures.
2. Addition of either Ca^{++} or MIX to such preparations caused significant increases in both EPP amplitude and quantal content.

To further investigate the possibility of Ca^{++} involvement in MIX action another series of MEPP experiments were conducted in Ca^{++} free perfusate. The purpose of these trials was to consider the difference between the source of Ca^{++} stores which were necessary to prevent EPP failures on the one hand and the Ca^{++} stores required for the maintenance of MEPPs on the other. According to Miledi and Thies (1967):

> MEPP release is not markedly dependent on external Ca^{++} unlike the immediate release evoked by a nerve terminal

and

> Spontaneous release of transmitter reflects the resting level of intracellular free Ca^{++} and the evoked release reflects the sum of the resting Ca^{++} and the Ca^{++} brought in by the action potential. (Alnaes and Rahamimoff, 1975).

In more precise terms, the aim of these experiments was to compare the possible Ca^{++} mobilization of extracellular Ca^{++} stores by MIX with the potential of MIX to mobilize intracellular Ca^{++} stores: the MIX induced reversal of EPP failures in low Ca^{++}/ high Mg^{++} experiments together with increased MEPP frequency in Ca^{++} free medium supports the presence of two Ca^{++} pools. The concept of dual Ca^{++} supply (Alnaes and Rahamimoff, 1975) was also supported by the observation that the elimination of external Ca^{++} from the bathing medium totally abolishes all EPPs while MEPPs remained present. The actions of MIX on EPP failures and MEPPs in Ca^{++} free medium suggests that MIX may mobilize Ca^{++} from both supplies or that enhanced intracellular Ca^{++} mobilization alone is sufficient to account for EPP failure reversal as well as elevated MEPP frequency. Of course there is always the possibility that MIX may act at some step beyond Ca^{++} involvement in the transmitter release sequence. This possibility, however, remains less likely since Ca^{++} involvement has been implicated in several other methylxanthine studies.

Still another important reason for investigating MEPPs in Ca^{++} free medium concerns the elimination of the effects of nerve terminal membrane potential shifts on spontaneous release parameters. The evidence for this concept is supported particularly from the studies (Borle, 1974; Alnaes and Rahamimoff, 1975) of the action of dicumarol on the nerve terminal mitochondrial Ca^{++}. This effect is quite complex.

Dicumarol, a compound which uncouples oxidative phosphorylation, is though to be capable of inhibiting Ca^{++} uptake ability into nerve terminal mitochondria in addition to causing a release of sequestrated mitochondrial Ca^{++}. At the nerve terminal, drug-induced intraneuronal release of Ca^{++} could account for the ten-fold increase in MEPP frequency. Metabolic inhibitors such as dicumarol may also exhibit nerve terminal depolarizing action since resting membrane potential is an energy dependent process. However, indirect evidence suggests that this is not the mechanism of action of dicumarol. Hubbard et al. (1969a), Del Castillo and Katz (1954b) and Liley (1956b) have documented that the actions of an agent on nerve terminal membrane potential are minimal in the presence of a lowered Ca^{++} perfusate:

> If a frequency change is definitely attributable to the agent of procedure under test, it is useful to repeat the experiment in the presence of a raised [Mg^{++}] or lowered [Ca^{++}] in the extracellular medium. There is good evidence at the nerve muscle junctions in vertebrates that the effects of depolarization or hyperpolarization of nerve terminals are reduced in these circumstances.

Alnaes and Rahamimoff (1975) further indicated that in the absence of external Ca^{++}, terminal depolarization has only a small effect on MEPP frequency. These investigators showed that when dicumarol was applied to neuromuscular junctions, MEPP frequency was significantly increased regardless of the presence or absence of Ca^{++} in the bathing medium. This finding eliminated the possibility that dicumarol produced its action by means of nerve terminal depolarization.

Accordingly, as MIX induced a significant increase in MEPP frequency in Ca^{++} free/high Mg^{++} medium, this compound acts by a mechanism other than nerve terminal depolarization.

The Cyclic AMP-Ca^{++} Relationship

At present, experimental evidence which links methylxanthines to cAMP involvement in the facilitation of neurotransmitter release is scant. However, in general, remarkable similarities seem to exist between Ca^{++} and cyclic nucleotide effects on synaptic transmission. These observations suggest that MIX may involve both Ca^{++} and cyclic AMP in the prejunctional facilitation of ACh release. The following discussion focuses mainly on indirect evidence for the functional relationship between cAMP and Ca^{++}.

In view of the well documented phosphodiesterase inhibitory actions of MIX (Beavo et al., 1965, Collier et al., 1975), observed effects of MIX may depend on increased levels of cAMP at the neuromuscular junction. The difficulty in establishing this relationship, concerns the inability to measure cAMP levels at viable neuromuscular junctions and, particularly, within nerve terminals (Miyamoto and Breckenridge, 1974). Consequently, evidence favoring cAMP involvement or the proposed link between cAMP and Ca^{++}, in the release of the transmitter is obtained by indirect methods. Such an indirect approach is also necessary in relating the actions of MIX to cAMP.

Evidence for the role of cAMP in the metabolic processes of numerous cell types was initially provided by Sutherland et al. (1968). These investigators demonstrated that catecholamines stimulated the synthesis of cAMP while theophylline prevented its breakdown. The

facilitation of neurotransmitter release in the presence of theophylline or epinephrine also implied the functional presence of cAMP at the neuromuscular junction (Breckenridge et al. 1967).

Subsequent investigations have shown that dibutyryl cAMP perfused onto neuromuscular junctions caused significant increases in MEPP frequency, quantal content, readily releasable stores, as well as depression of MEPP amplitude but insignificant effects on probability of release (Goldberg and Singer, 1969; Wilson, 1974a; Miyamoto and Breckenridge, 1974). With the exception of dibutyryl cAMP related increase in stores and unaffected probability of release, MIX actions are in agreement with the above results. In general the results obtained with other xanthines including caffeine and theophylline also resemble MIX findings.

In a variety of cell types, the requirement of both Ca^{++} and cAMP are necessary to produce a given effect. Furthermore, similarities between cAMP and Ca^{++} actions implied that both may be linked in the sequence of events leading to transmitter release. For example, in toad bladder, Ca^{++} ions are necessary for the transduction of information from the antidiuretic hormone (ADH) receptor to the adenyl cyclase (Bockaert et al. 1972). In this case, Ca^{++} ions are mandatory for the elevation of cAMP which in turn regulates Na^{+} and water transport (Berridge, 1975).

In another study, Ozawa and Ebashi (1967) have demonstrated the activation of phosphorylase-b-kinase by either Ca^{++} or by 3'5'-AMP in purufied muscle homogenate. However, 3×10^{-6} M cAMP, while elevating phosphorylase activation by 20% in the presence of Ca^{++}, fails to activate phosphorylase in the absence of Ca^{++}. These results led Rasmussen and Tenehause (1968) to suggest that 3'5'-AMP may alter the

permeability of cellular membranes to Ca^{++}. Furthermore, such an effect could account for both the positive inotropic effect of epinephrine via adenyl cyclase activation at the heart as well as the effects of epinephrine upon carbohydrate metabolism through increased cAMP production (Berridge, 1975).

MIX and theophylline were also shown to significantly elevate cAMP levels in pancreatic β-cells via phosphodiesterase inhibition (Montague and Cook, 1971). Insulin release, however, was dependent upon adequate external Ca^{++} supplies (Rubin, 1970) and a high $>$ 16.5 mM glucose concentration was a necessary prerequisite for induction of insulin secretion. At low glucose concentrations cAMP levels could be elevated but insulin release was unaffected.

Thus,

> The ability of intracellular cAMP to mobilize intracellular Ca^{++} would certainly account for the ability of dibutyryl cAMP or theophylline to restore the action of glucose in low Ca^{++} media (Brisson et al., 1972).

While cAMP by itself is not capable of stimulating insulin release, it is thought to act by sensitizing the β-cell to glucose by modulating Ca^{++} homeostasis (Berridge, 1975). It may then be reasoned that the ability of intracellular cAMP to mobilize intracellular Ca^{++} could account for the ability of MIX to restore EPPs in low Ca^{++} medium. However, caution must be excreted when comparing different cell types in the discussion of a common mechanism. Consideration of neurotransmitter release from nerve terminals can, however, be validly related to release of insulin from the pancreas as the pertinent results are consistent with a Ca^{++}-cAMP mechanism underlying both phenomena. Indeed, both

mechanisms appear to be similar if not identical since both cell types exhibit functional responses which are characterized by the following features:

(1) 3'5' cAMP is the probable regulator between stimulus and release (Hoffman, 1969; Wilson, 1974a; Rubin, 1970; Brisson et al.,1972);

(2) Ca^{++} is required in the external medium in order to make the appropriate stimulus effective;

(3) an elevated Mg^{++} in the external medium inhibits the response;

(4) a high concentration of K^{+} in the external medium functions as a nonspecific stimulus for release of either substance;

(5) the effect of K^{+} is also dependent upon the presence of Ca^{++};

(6) ouabain can stimulate either process (Rasmussen and Tenenhouse, 1968).

The results of Borle (1974) offer further support for cAMP related Ca^{++} release from mitochondria. Borle (1974) demonstrated that 1×10^{-7} to 3×10^{-6} M cAMP regulated cytoplasmic Ca^{++} by increasing mitochondrial Ca^{++} efflux rate in rat kidney, liver and heart mitochondria.

Despite the volume of evidence implicating the involvement of cAMP in synaptic transmission, some conflicting data yet remain. In an unpublished observation, Collier stated that neither theophylline nor dibutyryl cAMP had a measurable effect on superior cervical ganglion during short periods of stimulation (MacIntosh and Collier, 1976). Furthermore, adenosine which increased cAMP levels in brain slices (Sattin and Rall, 1970) reduced rather than increased quantum content of

the EPP of rat diaphragm (Ginsborg and Hirst, 1972). Such observations have allowed Ginsborg to conclude that the case for cAMP formation as a factor in evoked ACh release is not convincing at present. However, Miyamoto et al. (1969) explained that adenosine may compete with cAMP at the level of protein kinase or as an inhibitor of adenyl cyclase (McKenzie and Bär, 1973). Finally, the increase in cAMP in brain slices exposed to adenosine may result solely from glial cells (Clark and Perkins, 1972).

Evidence for cyclic nucleotide phosphodiesterase inhibiting actions of MIX and observed effects associated with increased cAMP levels support the idea of cyclic nucleotide modulation of neuromuscular transmission. As already mentioned, evidence favoring cAMP involvement or the relationship between cAMP and Ca^{++} in the release of transmitter is usually obtained by indirect methods. Such is the case in relating MIX to the overlapping actions of cAMP and Ca^{++} and is supported by the following results obtained in this laboratory and elsewhere:

A. MIX is a potent phosphodiesterase inhibitor as shown by the measurement of lipolysis in epididymal fat cells (Beavo et al. 1970).

B. Significant Ca^{++} mobilization is characterized by enhanced MEPP frequency, depressed MEPP amplitude (Mambrini and Benoit, 1963), elevated quantal content (Del Castillo and Katz, 1954c), reversal of EPP failures (Del Castillo and Engbaek, 1954), and increased p at the prejunctional nerve terminal (Hubbard et al., 1969).

C. Theophylline and dibutyryl cAMP have been shown to increase quantal content, mobilization rate and releasable store (Wilson,1974a). They increase EPP amplitude and MEPP frequency but depress MEPP amplitude (Goldberg and Singer, 1969; Miyamoto and Breckenridge, 1974).

D. Goldberg and Singer (1969) have shown that dibutyryl cAMP simultaneously increased MEPP frequency and quantal content in the presence of high Mg^{++}.

E. MIX dramatically duplicated all of the above actions with one exception: readily releasable stores were decreased rather than increased by MIX.

F. The prejunctional actions of theophylline, while possibly attributable to elevated cAMP levels via phosphodiesterase inhibition may also be explained by a caffeine-like action in altering Ca^{++} influx (Blinks et al. 1972). A similar functional explanation may also apply to MIX.

G. A number of investigations of many different cell types have linked cAMP to Ca^{++} in excitation-secretion coupling and excitation release. A statement by Berridge (1975) with respect to cAMP-Ca^{++} coupling is relevant:

> The primary intracellular second messenger in cells appears to be calcium, and the cyclic nucleotides seem to play a secondary role in either enhancing or dampening the calcium signal.

BIBLIOGRAPHY

Adrian, R.H., Chandler, W.K., and Hodgkin, A.L. (1970). Voltage clamp experiments in striated muscle fibers. J. Physiol. (London). 208, 607-644.

Albano, J., Bhoola, K.D., Heap, P.F., and Lemon, M.J.C. (1976). Stimulus-secretion coupling: role of cyclic AMP, cyclic GMP and calcium in mediating enzyme (Kallikrein) secretion in the submandibular gland. J. Physiol. (London). 258, 631-658.

Albuquerque, E.X., Barnard, E.A., Chiu, T.H., Lapa, A.J., Jolly, J.O., Jansson, S.E., Daly, J., and Witkop, B. (1973). Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: evidence from specific toxin reactions. Proc. Nat. Acad. Sci. (Washington). 70, 949-953.

Alnaes, E., and Rahamimoff, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. J. Physiol. (London). 248, 285-306.

Anderson, M.J., and Cohen, M.W. (1974). Flourescent staining of acetylcholine receptors in vertebrate skeletal muscle. J. Physiol. (London). 237, 385-400.

Auerbach, A. (1971). A spontaneous and evoked quantal transmitter release at the vertebrate central synapse. Nature, New Biol. 234, 181-183.

Auerbach, A., and Betz, W. (1971). Does curare affect transmitter release? J. Physiol. (London). 213, 691-705.

Axelsson, J., and Thesleff, S. (1958). Activation of the contractile mechanism in striated muscle. Acta Physiol. Scand., 44, 55-66.

Azhar, S., and Menon, K.M.J. (1977). Cyclic nucleotide phosphodiesterase from rat anterior pituitary. Characterization of multiple forms and regulation by protein activator and Ca^{++}. Eur. J. Biochem. 73, 73-82.

Banks, J., Kreider, J.W., Satok, C., and Davidson, E.A. (1975). Properties of acidic saccharides produced by B-16 melanoma cells treated with 1-methyl-3-isobutylxanthine. Cancer Res. 35, 2383-2389.

Barnes, J.M., and Duff, J.I. (1953). The role of cholinesterase at the myoneural junction. Brit. J. Industr. Med. 11, 11-19.

Barnard, E.A., Wieckowski, J., and Chiu, T.H. (1971). Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junction. Nature (London). 234, 207-209.

Barnard, E.A., Dally, J.O., Porter, C.W., and Albuquerque, E.X. (1975). The acetylcholine receptor and the ionic conductance modulation system of skeletal muscle. Exp. Neurol. 48, 1-28.

Barrett, E.T., and Barrett, J.N. (1976). Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurons. J. Physiol. (London). 255, 737-774.

Barstad, J.A.B., and Lilleheil, G. (1968). Transversally cut diaphragm preparation from rat. Arch. Int. Pharmacodyn. 175, 373-390.

Bear, R.S., and Schmitt, F.O. (1939). Electrolytes in the axoplasm of the giant nerve fibres of the squid. J. Cell. Comp. Physiol. 14, 205-215.

Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W., and Newman, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3'5'-monophosphate phosphodiesterase activity. Mol. Pharm. 6, 597-603.

Berg, D.K., Kelly, R.B., Sargent, P.B., Williamson, P., and Hall, Z.W. (1972). Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. Proc. Nat. Acad. Sci. (Washington). 69, 147-151.

Berg, P. (1956a). Acyl Adenylates: an enzymic mechanism of acetate activation. J. Biol. Chem. 222, 991-1013.

Berg, P. (1956b). Acyl Adenylates: the synthesis and properties of adenyl acetate. J. Biol. Chem. 222, 1015-1023.

Bernstein, T. (1902). Untersuchungen zur Thermodynamik der biolektrischen Strome. Pfluergers Arch. ges Physiol. 92, 521-562.

Berridge, M.J. (1975). The interaction of cyclic nucleotides and calcium in the control of cellular activity. In: advances in Cyclic Nucleotide Research. 6, 1-98. Greengard, P., and Robison, G.A. (eds).

Bianchi, C.P. and Shanes, A.M. (1959). Calcium influx in skeletal muscle at rest during activity and during potassium contraction. J. Gen. Physiol. 42, 803-815.

Bianchi, C.P. (1961). Caffeine effect on muscle calcium. J. Gen. Physiol. 44, 845-857.

Bianchi, C.P. (1962). Kinetics of radiocaffeine uptake and release in frog sartorius. J. Pharmacol. Exp. Ther. 138, 41-47.

Bianchi, C.P. (1975). Cellular pharmacology of contraction of skeletal muscle. In: Cellular Pharmacology of Excitable Tissues, Narahashi, T. (ed.). Thomas Books, Springfield, Illinois.

Birks, R.I., Huxley, H.E., and Katz, B. (1960). The fine structure of the neuromuscular junction of the frog. J. Physiol. (London). 150, 134-144.

Birks, R.I., Katz, B., and Miledi, R. (1960). Physiological and structural changes at the amphibian myoneural junction in the course of nerve degeneration. J. Physiol. (London). 150, 145-168.

Birks, R.I., and MacIntosh, F.C. (1961). Acetylcholine metabolism of a sympathetic ganglion. Can. J. Biochem. Physiol. 39, 787-827.

Blaber, L.C. (1970). The effect of facilitatory concentrations of decamethonium on the storage and release of transmitter at the neuromuscular junction in the cat. J. Pharmacol. Exp. Ther. 175, 664-672.

Blinks, J.R., Olson, C.B., Jewell, B.R., and Braveny, P. (1972). Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. Circ. Res. 30, 367.

Bockaert, J., Roy, C., and Jard, S. (1972). Oxytocin-sensitive adenylate cyclose in frog bladder epithelial cells. J. Biol. Chem. 247, 7073-7081.

Borle, A.B. (1974). Cyclic AMP stimulation of calcium efflux from kidney liver and heart mitochondria. J. Memb. Biol. 16, 221-236.

Bowman, W.C., and Nott, M.W. (1969). Actions of sympathomimetic amine and their antagonist on skeletal muscle. Pharmacol. Rev. 21, 27-72.

Boyd, I.A., and Martin, A.R. (1956). The endplate potential in mammalian muscle. J. Physiol. (London). 132, 74-91.

Boyle, P.J., and Conway, E.J. (1941). Potassium accumulation in muscle and associated changes. J. Physiol. (London). 100, 1-63.

Breckenridge, B.M.L. (1970). Cyclic AMP and Drug Action. Ann Rev. of Pharmacol. 10, 19-34.

Breckenridge, B.M.L. Burn, J.H., and Matschinsky, F.M. (1967). Theophylline, epinephrine and neostigmine facilitation of neuromuscular transmission. Proc. Nat. Acad. Sci. 57, 1893-1897.

Brisson, G.R., Nalaisse-Lagae, F., and Malaisse, W.J. (1972). The stimulus-secretion coupling of glucose-induced insulin release VII. A proposed site of action for adenosine 3', 5' cyclic monophosphate. J. Clin. Invest. 51, 232-241.

Brooks, V.B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin and membrane endplate potentials. J. Physiol. (London). 134, 264-277.

Brown, B.W. (1962). Estimation of the mean number of quanta per endplate potential. J. Pharmacol. Exp. Ther. 138, 327-328.

Brumlik, J., Jacobs, R., and Karczmar, A.G. (1973). Theophylline compounds and myasthenia gravis. II clinical report of nine cases. Clin. Pharmacol. Ther. 14, 380-385.

Burke, W. (1957). Spontaneous potentials in slow muscle fibers of the frog. J. Physiol. (London). 135, 511-521.

Burnstein, S., Gagnor, G., Hunter, S.A., and Maudsley, D.V. (1977). Elevation of prostaglandin and cyclic AMP levels by arachidonic acid in primary epithelial cell cultures of C3H mouse mammary tumors. Prostaglandins. 13, no. 1, 41-53.

Butcher, F.R., McBride, P.A., and Rudich, L. (1976). Cholinergic regulation of cyclic nucleotide levels, amylase release, and K^+ efflux from rat paratid glands. Mol. Cell. Endocrinol. 5, 243-254.

Butcher, R.W., and Sutherland, E.W. (1962). Adenosine 3'5' phosphate in biological materials. I Purification and properties of cyclic 3'5' nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3'5' phosphate in human urine. J. Biol. Chem. 237, 1244-1250.

Chang, C.C, and Lee, C.Y. (1963). Isolation of neurotoxins from the venom of Bungarus multicinctus and their modes of neuromuscular blocking action. Arch. Int. Pharmacodyn. 144, 241-257.

Chang, C.C., Chen, T.F., and Chuang, S.T. (1973). N, O-di and N, N, O-tri [^{3}H] acetyl α -bungarotoxins as specific labelling agents of cholinergic receptors. Brit. J. Pharmacol. 47, 147-160.

Changeux, J.P., Kasai, M., and Lee, C.Y. (1970). Use of a snake venom to characterize the cholinergic receptor protein. Proc. Nat. Acad. Sci. (Washington). 67, 1241-1247.

Cherrington, A.D., Assimacopoulos, F.D., Harper, S.C., Corbin, J.D., Park, C.R., and Exton, J.H. (1976). Studies on the α -adrenergic activation of hepatic glucose output. J. Biol. Chem. 251, 5209-5218.

Chlapowski, F.J. (1975). Effects of hormones on cyclic adenosine 3'5' monophosphate accumulation in transitional epithelium of the urinary bladder. J. Cyclic Nuc. Res. 1, 193-205.

Clark, R.B., and Perkins, J.P. (1972). The effect of adenosine on the formation of cyclic AMP (cAMP) in cultured human astrocytoma cells. Fed. Proc. 31, 513.

Claycomb, W.C. (1976). Poly (adenosine diphosphate ribose) polymerase activity and nicotinamide adenine dinucleotide in differentiating cardiac muscle. Biochem. J. 154, 387-393.

Cohen, I., Kita, H., and Van Der Kloot, W. (1974a). The intervals between miniature endplate potentials in the frog are unlikely to be independently or exponentially distributed. J. Physiol. (London). 236, 327-339.

Cohen, I., Kita, H., and Van Der Kloot, W. (1974b). The stochastic properties of spontaneous quantal release of transmitter at the frog neuromuscular junction. J. Physiol. (London). 236, 341-361.

Cohen, I., Kita, H., and Van Der Kloot, W. (1974c). Stochastic properties of spontaneous transmitter release at the crayfish neuromuscular junction. J. Physiol. (London). 236, 363-371.

Cole, K.S., and Hodgkin, A.L. (1939). Membrane and protoplasm resistance in the squid giant axon. J. Gen. Physiol. 22, 671-687.

Colquhaun, D., Rang, H.P., and Ritchie, J.M. (1974). The binding of tetrodotoxin and α -bungarotoxin to normal and denervated mammalian muscle. J. Physiol. (London). 240, 199-226.

Collier, H.O.J. (1974). The concept of the quasi-abstinence effect and its use in the investigation of dependence mechanisms. Pharmacol. 11, 58-61.

Collier, H.O.J., Francis, D.L. Henderson, G., and Schneider, C. (1974). Quasi morphine-abstinence syndrome. Nature. 249, 471-473.

Collier, H.O.J., and Francis, D.L. (1975). Morphine abstinence is associated with increased brain cyclic AMP. Nature. 255, 159-162.

Collier, H.O.J., and Francis, D.L. (1976). Proceedings: Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-1-methylxanthine (IBMX). Br. J. Pharmacol. 56, 382P.

Collier, H.O.J., Francis, D.L., and Roy, A.C. (1976). Opiates, cyclic nucleotides and xanthines. Adv. Biochem. Psychopharm. 15, 337-345.

Cooke, B.A., Lindh, M.L., and Janszen, F.H.A. (1976a). Correlation of protein kinase activation and testosterone production after stimulation of Leydig Cells with luteinizing hormone. Biochem. J. 160, 439-446.

Cooke, B.A., and Van Der Kemp, A.J.W.C.M. (1976b). Protein kinase activity in rat testis interstitial tissue, effect of luteinizing hormone and other factors. Biochem. J. 154, 371-378.

Cooke, J.D., Okamoto, K., and Quastel, D.M.J. (1973a). The role of calcium in depolarization-secretion coupling at the motor nerve terminal. J. Physiol. (London). 228, 459-497.

Cooke, J.D., Okamoto, K., and Quastel, D.M.J. (1973b). The role of calcium in depolarization-secretion coupling at the motor nerve terminal. J. Physiol. (London). 228, 377-405.

Corbin, J.D., and Keely, S.L. (1977). Characterization and regulation of heart adenosine 3'5' monophosphate-dependent protein kinase isozymes. J. Biol. Chem. 252, no. 3, 910-918.

Costa, M., Manen, C.A., and Russell, D.H. (1975). In viva activation of cAMP-dependent protein kinase by aminophylline and 1-methyl-3-isobutylxanthine. Biochem. Biophys. Res. Commun. 65, 75-81.

Couteaux, R. (1955). Localization of cholinesterases at neuro-muscular junctions. Int. Rev. Cytol. 4, 335-375.

Couteaux, R., and Nachmansohn, D. (1940). Changes of choline esterase at endplates of voluntary muscle following section of sciatic nerve. Proc. Soc. Exp. Biol. Med. 43, 177-181.

Couteaux, R. and Taxi, J. (1951). Recherches Histochimiques sur les cholinesterases synaptiques. C.R. Assoc. Anat. 70, 1030-1032.

Couteaux, R., and Taxi, J. (1952). Recherches histochimiques sur la distribution des activites cholinesterasiques au niveau de la synapse myoneurale. Arch. Anat. Micr. Morph. Exp. 41, 352-392.

Cowan, S.L. (1936). The initiation of all-or-none responses in muscle by acetylcholine. J. Physiol. (London). 88, 3P-5P.

Crossland, J., and Slater, P. (1968). The effect of some drugs on the "free and bound" acetylcholine content of rat brain. Brit. J. Pharmacol. 49, 322-327.

Csillik, B., and Knyihar, E. (1968). On the effect of motor nerve degeneration on the fine structural localization of esterases in the mammalian motor endplate. J. Cell. Sci. 3, 529-538.

Cubeddu, L., Barnes, E., and Weiner, N. (1975). Release of norepinephrine and dopamine-B-hydroxylase by nerve stimulation. An evaluation of a role for cyclic adenosine monophosphate. J. Pharmacol. Exp. Ther. 193, 105-127.

Curtis, H.J., and Cole, K. (1942). Membrane resting and action potentials from the squid giant axon. J. Cell. Comp. Physiol. 19, 135-144.

Dale, H.H., and Dudley, H.W. (1929). The presence of histamine and acetylcholine in the spleen of the ox and the horse. J. Physiol. (London). 68, 97-123.

Dale, H.H., Feldberg, W., and Vogt, M. (1936). Release of acetylcholine at voluntary motor nerve endings. J. Physiol. (London). 86, 358-380.

Dannies, P.S., Gautvik, K.M., and Tashjian, Jr., A.H. (1976). A possible role of cAMP in mediating the effects of thyrotropin-releasing hormone on prolactin release and on prolactin and growth hormone synthesis in pituitary cells in culture. Endocrinol. 98, 1147-1159.

Davson, H., and Danielli, J.F. (1943). The Permeability of Natural Membranes. Cambridge University Press, London.

DeRobertis, E., and Bennett, H.S. (1955). Some features of submicroscopic morphology of the synapses in frog and earthworm. J. Biophys. Biochem. Cytol. 1, 47-58.

DeRubertis, F.R., and Craven, P. (1977). Increased guanylate cyclose activity and guanosine 3'5'-monophosphate content in ethionine-induced hepatomas. Cancer Res. 37, pp15-21.

Del Castillo, J., and Engbaek, L. (1954). The nature of the neuromuscular block produced by magnesium. J. Physiol. (London). 124, 370-384.

Del Castillo, J., and Katz, B. (1954a). The effect of magnesium on the activity of motor nerve endings. J. Physiol. (London). 124, 553-559.

Del Castillo, J., and Katz, B. (1954b). Changes in endplate activity produced by pre-synaptic polarization. J. Physiol. (London). 124, 586-605.

Del Castillo, J., and Katz, B. (1954c). Quantal components of the end-plate potential. J. Physiol. (London). 124, 560-573.

Del Castillo, J., and Katz, B. (1955a). Local activity at a depolarized nerve-muscle junction. J. Physiol. (London). 128, 396-411.

Del Castillo, J., and Katz, B. (1955b). On the localization of acetylcholine receptors. J. Physiol. (London). 128, 157-181.

Del Castillo, J., and Katz, B. (1956). Biophysical aspects of neuromuscular transmission. Progr. Biophys. 6, 121-170.

Dodge, F.A., and Rahamimoff, R. (1967). Co-operative action of Ca ions in the transmitter release at the neuromuscular junction. J. Physiol. (London). 193, 419-432.

Drachman, D.B., (1978a). Myasthenia gravis (part 1). N. Engl. J. Med. 298, 136-142.

Drachman, D.B., (1978b). Myasthenia gravis (part 2). N. Engl. J. Med. 298, 186-192.

Drachman, D.B., Kao, I., Pestronk, A., and Toyka, K.V. (1976). Myasthenia gravis as a receptor disorder. Ann. N.Y. Acad. Sci. 274, 226-234.

Dretchen, K.L., Standaert, F.G., Skirboll, L.R., and Morgenroth, V.H. (1976). Evidence for a prejunctional role of cyclic nucleotides in neuromuscular transmission. Nature. 264, 79-81.

Dun, N.J., and Karczmar, A.G. (1977). A comparison of the effect of theophylline and cyclic adenosine 3':5'-monophosphate on the superior cervical ganglion on the rabbit by means of the sucrose gap method. J. Pharmacol. Exp. Ther. 202(1), 89-96.

Dun, N.J., Kaibara, I., and Karczmar, A.G. (1978). Direct postsynaptic membrane effect of dibutyryl cyclic GMP on mammalian sympathetic neurons. Neuropharmacol. 16(10), 715-717.

Duncan, C.J., and Statham, H.E. (1977). Interacting effects of temperature and extracellular calcium on the spontaneous release of transmitter at the frog neuromuscular junction. J. Physiol. (London). 268, 319-333.

Eccles, J.C., Katz, G., and Kuffler, S.W. (1941). Nature of the endplate potential in curarized muscle. J. Neurophysiol. 4, 362-387.

Eccles, J.C., Katz, B., and Kuffler, S.W. (1942). Effect of eserine on neuromuscular transmission. J. Neurophysiol. 5, 211-230.

Eccles, J.C., and Oconnor, W.J. (1939). Responses which nerve impulses evoke in mammalian striated muscles. J. Physiol. (London). 97, 44-102.

Edwards, C., and Ikeda, K. (1962). Effect of 2-PAM and succinylcholine on neuromuscular transmission in the frog. J. Pharmacol. Exp. Therap. 138, 322-327.

Elias, E.R. (1972). Histological and histochemical studies of red and white mammalian muscles, anterior horn cells and related bouton terminaux following denervation and reinervation. Ph.D. Thesis, University of London.

Ellman, G.L., Courtney, K.G., Andres, V., and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Elmqvist, D., Hofmann, W.W., Kugelberg, J., and Quastel, D.M.J. (1964). An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. J. Physiol. (London). 174, 417-434.

Elmqvist, D., and Feldman, D.S. (1965). Calcium dependence of spontaneous acetylcholine release of mammalian motor nerve terminals. J. Physiol. (London). 181, 487-497.

Elmqvist, D., and Quastel, D.M.J. (1965a). Presynaptic action of hemicholinium at the neuromuscular junction. J. Physiol. (London). 177, 463-482.

Elmqvist, D., and Quastel, D.M.J. (1965b). A quantitative study of endplate potentials in human muscle. J. Physiol. (London). 178, 505-529.

Erneux, C., VanSande, J., Dumont, J.E., and Boeynaems, J.M. (1977). Cyclic nucleotide hydrolysis in the thyroid gland. Eur. J. Biochem. 72, 137-147.

Ewald, D.A. (1976a). Potentiation of postjunctional cholinergic sensitivity of rat diaphragm muscle by high-energy-phosphate adenine nucleotides. J. Memb. Biol. 29, 47-65.

Ewald, D.A. (1976b). Modulation of postjunctional cholinergic sensitivity of rat diaphragm muscle by cyclic adenosine monophosphate. J. Memb. Biol. 29, 67-79.

Falk, G., and Fatt, P. (1964). Linear electrical properties of striated muscle fibres observed with intracellular electrodes. Proc. Roy. Soc. (London). B. 160, 69-123.

Fatt, P. (1950). The electromotive action of acetylcholine at the motor endplate. J. Physiol. (London). 11, 408-422.

Fatt, P., and Katz, B. (1950). Membrane potentials at the motor endplate. J. Physiol. (London). 11, 46P-47P.

Fatt, P., and Katz, B. (1951). An analysis of the endplate potential recorded with an intracellular electrode. J. Physiol. (London). 115, 320-370.

Fatt, P. and Katz, B. (1952). Spontaneous subthreshold activity at the motor nerve ending. J. Physiol. (London). 117, 109-128.

Ferry, C.B., and Marshall, A.R. (1971). Electrophysiological estimation of inhibition of acetylcholinesterase with normal transmitter release. J. Physiol. (London). 218, 66P-67P.

Flacke, W. (1973). Treatment of myasthenia gravis. N. Engl. J. Med. 288, 27-31.

Francis, D.L., Roy, A.C., and Collier, H.O.J. (1975). Morphine abstinence and quasiabstinence effects after phosphodiesterase inhibitors and naloxone. Life Sci. 16, 1901-1906.

Gage, P.W., and Hubbard, J.I. (1965). Evidence for a Poisson distribution of miniature endplate potentials and some implications. Nature (London). 208, 395-396.

Gallagher, J.P. (1972). PhD. dissertation. Action of Catechol and Phenol. Loyola University.

Gallagher, J.P. and Shinnick-Gallagher, P. (1977). Cyclic nucleotides injected intracellularly into rat superior cervical ganglion cells. Science. 198, 851-852.

Garbers, D.L., and Hardman, J.G. (1976). Effects of egg factors on cyclic nucleotide metabolism in sea urchin sperm. J. Cyclic Nuc. Res. 2, 59-70.

Ginsborg, B.L. and Hirst, G.D.S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. J. Physiol. (London). 224, 629-645.

Ginsborg, B.L., and Jenkinson, D.H. (eds.) (1976). Transmission of impulses from nerve to muscle. In: Handbuch der Experimentellen Pharmakologie Erganzungswerk; Neuromuscular Junction. 42, 299-364. Berlin-Gottingen-Heidelberg, Springer.

Goldberg, A.L., and Singer, J.J. (1969). Evidence for a role of cyclic AMP in neuromuscular transmission. Proc. Nat. Acad. Sci. 64, 134-141.

Goldman, D.E. (1943). Potential impedance and rectification in membranes. J. Gen. Physiol. 27, 37-60.

Goodman, L.S., and Gilman, A. (eds.) (1975). The Pharmacological Basis of Therapeutics. 5th ed., Macmillan, New York.

Gordon, T. (1976). The effect of external calcium and magnesium ions on the response of denervated muscle to acetylcholine. J. Physiol. (London). 255, 575-586.

Goutier, R. (1949). Action des methylxanthines sur la transmission neuromusculaire. Arch. Intern. Physiol. 57, 185-200.

Greengard, P., and Kebabian, J.W. (1974). Role of cyclic AMP in synaptic transmission in the mammalian peripheral nervous system. Fed. Proc. 33, 1059-1067.

Grill, V., Asplund, K., Hellerstrom, C., and Cerasi, E. (1975). Decreased cyclic AMP and insulin response to glucose in isolated islets of neonatal rats. Diabetes. 24, 746-752.

Hahn, H.J., and Gottschling, H.D. (1976). Somatostatin-induced inhibition of insulin secretion by isolated pancreatic rat islets prepared by micro-dissection or collagenase digestion. Diab. Metab. 2, 107-111.

Hamprecht, B. (1973). Stimulation by prostaglandin E_1 of adenosine 3'5'-cyclic monophosphate formation in neuroblastoma cells in the presence of phosphodiesterase inhibitors. F.E.B.S. Lett. 34, no. 1, 85-89.

Haring, H.U., Renner, R., and Kepp, K.D. (1976). Hormone control of cyclic AMP turnover in isolated fat cells. Mol. Cell. Endocrinol. 5, 295-302.

Hartzell, H.C., and Fambrough, D.M. (1962). Acetylcholine receptors: Distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J. Gen. Physiol. 60, 248-262.

Haugaard, N., and Hess, M.E. (1966). The influence of catecholamines on heart function and phosphorylase activity. Pharmacol. Rev. 18, 197-203.

Hellman, B. (1975). The significance of calcium for glucose stimulation of insulin release. Endocrin. 97, 392-398.

Hellman, B. (1976). Effects of starvation and Ca^{++} on glucose-induced accumulation of cyclic 3'5'-AMP in pancreatic islets. Experentia. 32, 155-157.

Herz, R., and Weber, A. (1965). Caffeine inhibition of Ca^{++} uptake by muscle reticulum. Fed. Proc. 24, 208.

Heuser, J.E., and Reese, T.S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell. Biol. 57, 315-344.

Hille, B. (1970). Ionic channels in nerve membranes. Prog. Biophys. Mol. Biol. 21, 1-32.

Hobbiger, F. (ed.) (1976). Pharmacology of anticholinesterase drugs, in: Handbuch der Experimentellen Pharmakalogie Erganzungswerk; Neuromuscular Junction. 42, 487-581. Berlin-Gottingen-Heidellberg, Springer.

Hodgkin, A.L. (1937). Evidence for electrical transmission in nerve. J. Physiol. (London). 90, 183-232.

Hodgkin, A.L. (1958). The Croonian Lecture: Ionic movements and electrical activity in giant nerve fiberes. Proc. Roy. Soc. (London) B. 148, 1-37.

Hodgkin, A.L. (1976). Chance and design in electrophysiology: an informal account of certain experiments on nerve carried out between 1934 and 1952. J. Physiol. (London). 263, 1-21.

Hodgkin, A.L., and Horowicz, P. (1959). Movements of sodium and potassium in single muscle fibers. J. Physiol. (London). 145, 403-432.

Hodgkin, A.L., and Huxley, A.F. (1939). Action potentials recorded from inside a nerve fibre. Nature. 144, 710-711.

Hodgkin, A.L., and Huxley, A.F. (1945). Resting and action potentials in single nerve fibers. J. Physiol. (London). 104, 176-195.

Hodgkin, A.L., and Huxley, A.F. (1952a). Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo. J. Physiol. (London). 116, 449-472.

Hodgkin, A.L., and Huxley, A.F. (1952b). The components of membrane conductance in the giant axon of Loligo. J. Physiol. (London). 116, 473-496.

Hodgkin, A.L., and Huxley, A.F. (1952c). The dual effect of membrane potential on sodium conductance in the giant axon of Loligo. J. Physiol. (London). 116, 497-506.

Hodgkin, A.L., and Huxley, A.F. (1952d). A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. (London). 117, 500-544.

Hodgkin, A.L., Huxley, A.F., and Katz, B. (1952). Measurement of current-voltage relations in the membrane of the giant axon of Loligo. J. Physiol. (London). 116, 424-448.

Hodgkin, A.L., and Katz, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol. (London). 108, 37-77.

Hodgkin, A.L., and Keynes, R.D. (1955). Active transport of cations in giant axons from Sepia and Loligo. J. Physiol. (London). 128, 28-60.

Hodgkin, A.L., and Martin, K. (1965). Choline uptake by giant axons of Loligo. J. Physiol. (London). 174, 26P-27P.

Hodgkin, A.L., and Rushton, W.A.H. (1946). The electrical constants of a crustacean nerve fiber. Proc. Roy. Soc. (London). B, 133, 444-479.

Hofmann, W.W. (1969). Caffeine effects on transmitter depletion and mobilization at motor nerve terminals. Am. J. Physiol. 216, 621-629.

Hofmann, W.W., Parsons, R.L., and Feigen, G.A. (1966). Effects of temperature and drugs on mammalian motor nerve terminals. Amer. J. Physiol. 211, 135-140.

Hornbrook, K.R., and Brody, T.M. (1963a). The effect of catecholamines on muscle glycogen and phosphorylase activity. J. Pharmacol. Exp. Ther. 140, 295-307.

Hornbrook, K.R., and Brody, T.M. (1963b). Phosphorylase activity in rat liver and skeletal muscle after catecholamines. Biochem. Pharmacol. 12, 1407-1415.

Howell, J.N. (1969). A lesion of the transverse tubules of skeletal muscle. J. Physiol. (London). 201, 515-533.

Huang, M., Shimizu, H., and Daly, J.W. (1972). Accumulation of cyclic adenosine monophosphate in incubated slices of brain tissue. 2. Effects of depolarizing agents, membrane stabilizers, phosphodiesterase inhibitors and adenosine analogues. J. Med. Chem. 15, 462-466.

Hubbard, J.I. (1970). Mechanisms of transmitter release. Prog. Biophys. Mol. Biol. 21, 33-124.

Hubbard, J.I., and Jones, S.F. (1973). Spontaneous quantal transmitter release: A Statistical analysis and some implications. J. Physiol. (London). 232, 1-21.

Hubbard, J.I., and Willis, W.D. (1962). Hyperpolarization of mammalian motor nerve terminals. J. Physiol. (London). 163, 115-137.

Hubbard, J.I., and Wilson, D.F. (1973). Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of d-tubocurarine. J. Physiol. (London). 228, 307-325.

Hubbard, J.I., Jones, S.F., and Landau, E.M. (1968a). On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. J. Physiol. (London). 194, 355-380.

Hubbard, J.I., Jones, S.F., and Landau, E.M. (1968b). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. J. Physiol. (London). 196, 75-87.

Hubbard, J.I., Jones, S.F., and Landau, E.M. (1971). The effect of temperature change upon transmitter release, facilitation and posttetanic potentiation. J. Physiol. (London). 216, 591-609.

Hubbard, J.I., Llinas, R., and Quastel, D.M.J. (1969a). Electrophysiological Analysis of Synaptic Transmission. Williams and Wilkins, Baltimore.

Hubbard, J.I., Wilson, D.F., and Miyamoto, M. (1969b). Reduction of transmitter release by d-tubocurarine. Nature. 233, 531-533.

Huidobro, F. (1945). A comparative sutdy of the effectiveness of 1, 3, 7 trimethylxanthine and certain dimethylxanthines (1, 3 dimethylxanthine and 3, 7 dimethylxanthine) against fatigue. J. Pharmacol. Exp. Ther. 84, 380-386.

Hunt, R. (1901). Further observations on the blood pressure lowering bodies in extracts of the suprarenal gland. Am. J. Physiol. 5, 5-7.

Hunt, R., and Taveau, R. (1906). On the physiological action of certain choline derivatives and new methods for detecting choline. Brit. Med. J. 2, 1788-1791.

Huxley, A.F., and Stampfli, R. (1951). Effect of potassium and sodium on resting and action potentials of single myelinated nerve fibers. J. Physiol. (London). 112, 496-508.

Isaacson, A., and Sandow, A. (1967). Caffeine effects on radiocalcium movement in normal and denervated rat skeletal muscle. J. Pharmacol. Exp. Ther. 155, 376-388.

Jacobs, R., Brumlik, J., and Karczmar, A.G. (1971). Theophylline preparations in the treatment of myasthenia gravis. Trans. Am. Neurol. Ass. 96, 158-161.

Jacobs, R., Karczmar, A.G., and Brumlik, J. (1973). Theophylline compounds and myasthenia gravis 1. in vitro studies. Clin. Pharmacol. Ther. 14, 347-379.

Jacobs, R.S., and McNiece, D.M. (1977). Motor nerver terminal facilitatory action of SQ 20009: an inhibitor of cyclic nucleotide phosphodiesterase. J. Pharm. Exp. Therap. 202 (2), 404-410.

Jacobs, R.S., and Shinnick, P.L. (1973). Facilitatory sites of action of theophylline in isolated cat tenuissimus muscle. Neuropharmacol. 12, 885-895.

Jenkinson, D.H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. J. Physiol. (London). 138, 434-444.

Jenkinson, D.H., Stamenovic, G.Q. and Whittacker, B.D.L. (1968). The Effect of Noradrenaline on the Endplate Potential in Twitch Fibres of the Frog. J. Physiol. (London). 195, 743-754.

Johnson, E.W., and Wernig, A. (1971). The binomial nature of transmitter release at the crayfish neuromuscular junction. J. Physiol. (London). 218, 757-767.

Jolly, F. (1895). Pseudoparalysis myasthenia. Neurol. Central. 1, 3436.

Jones, S.F., and Kwanbunbumpen, S. (1970a). The effects of nerve stimulation and hemicholinium on quantal transmitter release at the mammalian neuromuscular junction. J. Physiol. (London). 207, 31-50.

Jones, S.F., and Kwanbunbumpen, S. (1970b). Some effects of nerve stimulation and hemicholinium on quantal transmitter release at the mammalian neuromuscular junction. J. Physiol. (London). 207, 51-61.

Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K., and Miyamoto, E. (1975). Multiple cyclic nucleotide phosphodiesterase activities from rat tissues and occurrence of a calcium-plus-magnesium-ion cependent phosphodiesterase and its protein activator. Biochem. J. 146, 109-120.

Kalix, P., McAfee, D.A., Schorderet, M., and Greengard, P. (1974). Pharmacological analysis of snyaptically mediated increase in cyclic adenosine monophosphate in rabbit superior cervical ganglion. J. Pharmacol. Exp. Ther. 188, 676-687.

Kanagasuntheram, P., and Randle, P.J. (1976). Calcium metabolism and amylase release in rat parotid acinar cells. Biochem. J. 160, 547-564.

Kao, C.Y. (1966). Tetroditoxin, saxitoxin and their significance in the early study of excitation phenomenon. Pharmacol. Rev. 18. 997-1049.

Karczmar, A.G. (1967). Neuromuscular pharmacology. Ann. Rev. Pharmacol. 7, 241-276.

Katz, B. (1937). The time course of the excitatory disturbance in nerve following a short shock. J. Physiol. (London). 6P-8P.

Katz, B. (1938). Experimental evidence for a non-conducted response of nerve to subthreshold stimulation. Proc. Roy. Soc. (London). 124, B, 244-276.

Katz, B. (1942). Impedence changes in frog muscle associated with electronic and endplate potentials. J. Neurophysiol. 5, 169-184.

Katz, B. (1947). The effect of electrolyte deficiency on the rate of conduction in a single nerve fibre. J. Physiol. (London). 106, 411-417.

Katz, B. (1948). The electrical properties of the muscle fibre membrane. Proc. Roy. Soc. (London). 135, B, 506-534.

Katz, B. (1962). The transmission of impulses from nerve to muscle and the subcellular unit of synaptic action. Proc. Roy. Soc. (London). 155, B, 455-477.

Katz, (1966). Nerve, Muscle and Synapse. McGraw-Hill, New York.

Katz, B., and Miledi, R. (1961) The localized action of "endplate drugs" in the twitch fibers of the frog. J. Physiol. (London). 155, 399-415.

Katz, B. and Miledi, R. (1965a). Propagation of electric activity in motor nerve terminals. Proc. Roy. Soc. (London). 161, B, 453-482.

Katz, B., and Miledi, R. (1965b). The measurement of synaptic delay and the time course of acetylcholine release at the neuro-muscular junction. Proc. Roy. Soc. (London). 161, B, 483-495.

Katz, B., and Miledi, R. (1965c). The effect of calcium on acetylcholine release from the motor nerve terminals. Proc. Roy. Soc. (London). 161, B, 496-503.

Katz, B., and Miledi, R. (1969). Spon taneous and evoked activity of motor nerve endings in calcium ringer. J. Physiol. (London). 203, 689-706.

Katz, B., and Miledi, R. (1970). Further study of the role of calcium in synaptic transmission. J. Physiol. (London). 207, 789-801.

Katz, B., and Miledi, R. (1971). The effect of prolonged depolarization on synaptic transfer in the stellate ganglion of the squid. J. Physiol. (London). 216, 503-512.

Katz, B., and Miledi, R. (1973a). The characteristics of "endplate noise" produced by different depolarizing drugs. J. Physiol. (London). 230, 707-717.

Katz, B., and Miledi, R. (1973b). The binding of acetylcholine to receptors and its removal from the synaptic cleft. J. Physiol. (London). 231, 549-574.

Katz, B., and Miledi, R. (1973c). The effect of α-bungarotoxin on acetylcholine receptors. Brit. J. Pharmacol. 49, 138-139.

Katz, B., and Thesleff, S. (1957). On the factors which determine the amplitude of the miniature endplate potential. J. Physiol. (London). 137, 267-278.

Keely, S.L., Corbin, J.D., and Park, C.R. (1975). Regulation of adenosine 3'5' monophosphate dependent protein kinase, regulation of the heart enzyme by epinephrine, glucagon, insulin and 1-methyl-3-isobutylxanthine. J. Biol. Chem. 250, 4832-4840.

Kelly, L.A., and Butcher, R.W. (1975). Cyclic AMP metabolism in human epidermoid carcinoma (HE_p-2) cells. Metab. Clin. Exp. 24, 359-368.

Kempen, H.J.M., DePont, J.J.H.H.M., and Bonting, S.L. (1977a). Rat pancreatic adenylate cyclase. IV. Effect of hormones and other agents on cyclic AMP level and enzyme release. Biochim. Biophys. Acta. 496, 65-76.

Kempen, H.J.M., DePont, J.J.H.H.M., and Bonting, S.L. (1977b). Rat pancrease adenylate cyclase. V. Its presence in isolated rat pancreatic acinar cells. Biochim. Biophys. Acta. 496, 521-531.

Keynes, R.D. (1951). The ionic movements during nervous activity. J. Physiol. (London). 114, 119-150.

Keynes, R.D. (1963). Chloride in the squid giant axon. J. Physiol. (London). 169, 690-705.

Keynes, R.D., and Lewis, P.R. (1951). The sodium and potassium content of cephalopod nerve fibres. J. Physiol. (London). 114, 151-182.

Koelle, G.B., (1950). The histochemical differentiation of types of cholinesterases and their localizations in tissues of the cat. J. Pharmacol. Exp. Ther. 100, 158-179.

Koelle, G.V. (1951). The elimination of enzymatic diffusion artifacts in histochemical localization of cholinesterases and a survey of their cellular distribution. J. Pharmacol. Exp. Ther. 103, 153-171.

Koelle, G.V. (ed.) (1963). Cytological distributions and physiological functions of cholinesterase. In: Handbuch der Experimentellen Pharmakologie, Erganzungswerk; Cholinesterases and anticholinesterase agents. 187-298, Springer-Verlag, Berlin-Gottingen-Heidelberg.

Koelle, G.V., Davis, R., and Gromadzki, C.G. (1967). Electron microscopic localization of cholinesterases by means of gold salts. Ann. N.Y. Acad. Sci. 144, 613-625.

Koelle, G.V., Friedenwald, J.S. (1949). A histochemical method for localizing cholinesterase activity. Proc. Soc. Exp. Biol. (N.Y.). 70, 617-622.

Kriebel, M.E., and Gross, C.E. (1974). Multimodal distribution of frog miniature endplate potentials in adult denervated and tadpole leg muscle. J. Gen. Physiol. 64, 85-103.

Kriebel, M.E., and Stolper, D.R. (1975). Non-poisson distribution in time of small and large-mode miniature endplate potentials. Am. J. Physiol. 229, 1321-1329.

Kriebel, M.E., Llados, F., and Mattheson, D.R. (1976). Spontaneous subminiature endplate potentials in mouse diaphragm muscle: evidence for synchronous release. J. Physiol. (London). 262, 553-581.

Krnjevic, K., and Straughan, D.W. (1964). The release of acetylcholine from denervated rat diaphragm. J. Physiol. (London). 170, 371-378.

Kuba, K. and Nishi, S. (1976). Rhythmic hyperpolarizations and depolarizations of sympathetic ganglion cells induced by caffeine. J. Neurophysiol. 39, 547-563.

Kuffler, S. (1943). Specific excitability of the endplate region in normal and denervated muscle. J. Neurophysiol. 6, 99-110.

Landowne, D., Potter, L.T., and Terrar, D.A. (1975). Structure-function relationship in excitable membranes. Ann. Rev. Physiol. 37, 485-508.

Langley, J.M. (1909). On the contraction of muscle, chiefly in relation to the presence of "receptive substances." IV. The effect of curare and of some other substances on the nicotine response of the sartorious and gastrocnemius muscle of the frog. J. Physiol. (London). 39, 235-295.

Lee, C.Y. (1972). Chemistry and pharmacology of polypeptide toxins in snake venoms. Ann. Rev. Pharmacol. 12, 265-286.

Leichtling, B.H., Drotar, A.M., Ortmann, R., and Perkins, J.P. (1976). Growth of astrocytoma cells in the presence of prostaglandin E_1: Effect on the regulation of cyclic AMP metabolism. J. Cyclic Nuc. Res. 2, 89-97.

Lewis, P.R. (1961). The effect of varying the conditions in the Kolle technique. In: histochemistry of cholinesterase, Symposium, Basel. Bibl. Anat. 2, 11-20.

Liley, A.W. (1956a). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. (London). 132, 650-666.

Liley, A.W. (1956b). The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. J. Physiol. (London). 134, 427-443.

Liley, A.W. (1957). Spontaneous release of transmitter substance in multiquantal units. J. Physiol. (London). 136, 595-605.

Ling, G., and Gerard, R.W. (1949). The normal membrane potential of frog sartorius fibers. J. Cell. Comp. Physiol. 34, 383-396.

Liittgau, H.C., and Oetliker, H. (1968). The action of caffeine on the activation of the contractile mechanism in striated muscle fibers. J. Physiol. (London). 194, 51-74.

Loewi, O. (1921). Uber Uber Humorale Ubertragbarkeit der Herznerven-wirkung. I. Mitteilung. Pfluegers Arch. Ges. Physiol. 189, 239-242.

Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenon reagent. J. Biol. Chem. 193, 265-275.

Lunt, G.G., Stefani, E., and DeRobertis, E. (1971). Increased Incorporation of [G-^{3}H] leucine into a possible "receptor" proteolipid in denervated muscle in vivo. J. Neurochem. 18, 1545-1553.

MacIntosh, F.C., Birks, R.I., and Sastry, P.B. (1956). Pharmacological inhibition of acetylcholine synthesis. Nature (London). 178, 1181.

MacIntosh, F.C., Birks, R.I., and Sastry, P.B. (1958). Mode of action of an inhibition of acetylcholine synthesis. Neurol. 8, supp. 1, 70-91.

MacIntosh, F.C., and Collier, B. (eds.) (1976). Neurochemistry of cholinergic terminals. In: Hanbuch der Experimentellen Pharmakologie Erganzungswerk; Neuromuscular Junction. 42, 98-228. Springer-Verlag, Berlin-Gottingen-Heidelberg.

Magazanik, L.G., and Vyskocil, F. (1970). Dependence of acetylcholine desensitization of the membrane potential of frog muscle fibre and on the ionic changes in the medium. J. Physiol. (London). 210, 507-518.

Magleby, K.L., and Stevens, C.F. (1972). The effect of voltage on the time course of endplate currents. J. Physiol. (London). 233, 151.

Mambrini, J., and Benoit, P.R. (1963). Action de la Cafeine sur les jonctions neuro-musculaires de la genoiulle. Comptes Rendus. Soc. Biol. 157, 1373-1377.

Marnay, A., and Nachmansohn, D. (1938). Cholinesterase in voluntary muscle. J. Physiol. (London). 92, 37.

Martin, A.R. (1955). A further study of the statistical composition of the endplate potential. J. Physiol. (London). 130, 114-122.

Martin, A.R. (1966). Quantal nature of synaptic transmission. Physiol. Rev. 46, 51.

Matthews, G., and Wickelgren, W.O. (1977). On the effect of calcium on the frequency of miniature endplate potentials at the frog neuromuscular junction. J. Physiol. (London). 266, 91-101.

McAfee, D.A., Schorderet, M., and Greengard, P. (1972). Adenosine 3'5'-monophosphate: Electrophysiological evidence for a role in synaptic transmission. Science. 178, 310-312.

McKenzie, S.G., and Bär, H.P. (1973). On the mechanism of adenyl cyclase inhibition by adenosine. Can. J. Physiol. Pharmacol. 51, 190.

McKenzie, S.G., and Frew, R., and Bar, H.P. (1977a). Characteristics of the relaxant response of adenosine and its analogs in intestinal smooth muscle. Eur. J. Pharmacol. 41, 183-192.

McKenzie, S.G., Frew, R., and Bar, H.P. (1977b). Effects of adenosine and related compounds on adenylate cyclase and cyclic AMP levels in smooth muscle. Eur. J. Pharmacol. 41, 193-203.

McLachlan, E.M. (1978). The Statistics of Transmitter Release at Chemical Synapses. Int. Rev. Physiol. 17, 49-117.

McNeill, J.H., Brenner, M.J., and Muschek, L.D. (1973). Interaction of four methylxanthine compounds and norepinephrine on cardiac phosphorylase activation and cardiac contractility. Recent Advan. Stud. Cardiac. Struct. Metab. 3, 261-273.

Meer, C. VanDer, and Meeter, E. (1956). The mechanism of action of anticholinesterases. II. The effect of diisopropylflourophosphate (DFP) on the isolated rat phrenic nerve-diaphragm preparation. A. Irreversible Effects. Acta. Physiol. Pharmacoll Neerl. 4, 454-471.

Meunier, J.C., Olsen, R.W. and Changeaux, J.P. (1972). Studies of the cholinergic receptor protein from electrophorus electricus. FEBS Lett. 24, 63-68.

Miledi, R., Molinoff, P., and Potter, L.T. (1971). Isolation of cholinergic receptor protein of torpedo electric tissue. Nature. (London). 229, 554-557.

Miledi, R., and Potter, L.T. (1971). Acetylcholine receptors in muscle fibres. Nature. (London). 233, 599-603.

Miledi, R., and Theis, R.E. (1967). Post-tetanic increase in frequency of miniature endplate potentials in calcium-free solutions. J. Physiol. (London). 192, 54P-55P.

Mira-Moser, F., Schofield, J.C., and Orci L. (1976). Modifications in the release of rat growth hormone in vitro and the morphology of rat anterior pituitaries incubated in various ionophores. Eur. J. Clin. Invest. 6, 103-111.

Mittag, T., Kornfeld, P., Tormay, A. and Wood, C. (1976). Detection of anti-acetylcholine receptor factors in serum and thymus from patients with myasthenia gravis. N. Eng. J. Med. 294, 691-694.

Miyamoto, E., Kuo, J.F., and Greengard, P. (1969). Cyclic nucleotide-dependent protein kinases. III. Purification and properties of adenosine 3', 5'-monophosphate-dependent protein kinase from bovine brain. J. Biol. Chem. 244, 6395-6402.

Miyamoto, M.D., and Breckenridge, F.McL. (1974). A cyclic adenosine monophosphate link in the catecholamine enhancement of transmitter release at the neuromuscular junction. J. Gen. Physiol. 63, 609-624.

Montague, W., and Cook, J.E. (1971). Role of adenosine 3'5' cyclic monophosphate in the regulation of insulin release by isolated rat islets of langerhans. Biochem. J. 122, 115-120.

Nachmansohn, D., and Machado, A.L. (1943). The formation of acetylcholine. A new enzyme, "choline acetylase." J. Neurophysiol. 6, 397-403.

Namba, T., and Grob, D. (1968). Cholinesterase activity of the motor endplate in isolated muscle membrane. J. Neurochem. 15, 1445-1454.

Nastuk, W.L. (1953). Membrane potential changes at a single muscle endplate produced by transitory application of acetylcholine with an electrically controlled microjet. Fed. Proc. 12, 102.

Nastuk, W.L. (1966). Fundamental aspects of neuromuscular transmission. Ann. N.Y. Acad. Sci. 135, 110-135.

Nauss, K.M., and Davies, R.E. (1966). Changes in phosphate compounds during the development and maintenance of rigor mortis. J. Biol. Chem. 241, 2918-2922.

O'Brien, R.D., Elderfrawi, M.E., and Elderfrawi, A.T. (1972). Isolation of acetylcholine receptors. Ann. Rev. Pharmacol. 12, 19-34.

Ochs, S., and Mukherjee, A.K. (1959). Action of acetylcholine, choline and d-tubocurarine on the membrane of frog sartorius muscle. Am. J. Physiol. 196, 1191-1196.

Onodera, K. (1973). Effect of caffeine on the neuromuscular junction of the frog and its relation to external calcium concentration. Jap. J. Physiol. 23, 587-597.

Otsuka, M., Endo, M., and Nonomura, Y. (1962). Presynaptic nature of neuromuscular depression. Jap. J. Physiol. 12, 573-584.

Ozawa, E., and Ebashi, S. (1967). Requirement of Ca ion for the stimulating effect of cyclic 3', 5'-AMP on muscle phosphorylase b kinase. J. Biochem. 62, 285-286.

Pariza, M.W., Butcher, F.R., Kletzien, R.F., Becker, J.E., and Potter, V.R. (1976). Induction and decay of glucagon-induced amino acid transport in primary cultures of adult rat liver cells, paradoxical effects of cycloheximide and puromycin. Proc. Nat. Acad. Sci. (N.Y.). 73 no. 12, 4511-4515.

Passonneau, J.V., and Crites, S.K. (1976). Regulation of clycogen metabolism in astrocytoma and neuroblastoma cells in culture. J. Biol. Chem. 241, 2015-2022.

Peper, K., and McMahon, U.J. (1972). Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. Proc. Roy. Soc. 181, B, 431-440.

Perry, W.L.M. (1953). Acetylcholine release in the cats superior cervical ganglion. J. Physiol. (London). 119, 439-454.

Peytremann, A., Nicholson, W.E., Liddle, G.W., Hardman, J.G., and Sutherland, E.W. (1973). Effects of methylxanthines on adenosine 3'5' monophosphate and corticasterone in rat adrenal. Endocrinol. 92, 525-530.

Plattner, F., and Hinter, H. (1930). Die spaltung von acetylcholin durch organertrake und korperflussigkeiten. Pflug. Arch. Ges Physiol. 225, 19-25.

Porter, C.W., Chiu, T.H., Wieckowski, J., and Barnard, E.A. (1973). Types and locations of cholinergic receptor-like molecules in muscle fibers. Nature (London). New Biol. 241, 3-7.

Potter, L.T. (1970). Synthesis, storage and release of [^{14}C] acetylcholine in isolated rat diaphragm muscles. J. Physiol. (London). 206, 145-166.

Prince, W.T., Berridge, M.J., and Rasmussen, H. (1972). Role of calcium and adenosine 3', 5' cyclic monophosphate in controlling fly salivary gland secretion. Proc. Nat. Acad. Sci. (N.Y.). 69, 553-557.

Rabinovitch, A., Renold, A.E., and Cerasi, E. (1976). Decreased cyclic AMP and insulin responses to glucose in pancreatic islets of diabetic chinese hamsters. Diabetolog. 12, 581-591.

Raftery, M.A. (1973). Isolation of acetylcholine receptor-d-bungarotoxin complexes from torpedo californica electroplax. Arch. Biochem. Biophys. 154, 270-276.

Rang, H.P. (1975). Acetylcholine receptors. Quart. Rev. Biophys. 7, 283-399.

Rasmussen, H. (1970). Cell communication, calcium ion and cyclic adenosine monophosphate. Science. 170, 404-412.

Rasmussen, H., and Tenenhouse, A. (1968). Cyclid adenosine monophosphate, Ca^{++}, and membranes. Proc. Nat. Acad. Sci. 59, 1364-1370.

Renkin, E.M. (1961). Permeability of frog skeletal muscle cells to choline. J. Gen. Physiol. 44, 1159-1164.

Riker, W.F., and Okamoto, M. (1969). Pharmacology of motor nerve terminals. Ann. Rev. Pharmacol. 9, 173-208.

Rotshenker, S., and Rahamimoff, R. (1970). Neuromuscular synapse: stochastic properties of spontaneous release of transmitter. Science. 170, 648-649.

Rubin, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22, 389-428.

Rushton, W.A.H. (1932). A new observation in the excitation of nerve and muscle. J. Physiol. (London). 75, 16P-17P.

Rushton, W.A.H. (1937). The initiation of the nervous impulse. J. Physiol. (London). 90, 5P-6P.

Rushton, W.A.H. (1938). Initiation of the propagated disturbance. Proc. of the Roy. Soc. of London. 124, Ser. B. 210-243.

Sacks, L.E., and Thompson, P.A. (1975). Influence of methylranthines on sporulation of clostriduim perfringens cells. Spores. 6, 341-345.

Sandell, E.B. (1965). Colorimetric Determinationof Traces of Metals. 3rd ed. Interscience. Pub. Inc., New York.

Sandrow, A., Taylor, S.P., Isaacson, S., and Seguin, J.J. (1964). Electrochemical coupling in potentiation of muscular contraction. Science. 143, 577-579.

Sandow, A. (1965). Excitation-contraction coupling in skeletal muscle. Pharmacol. Rev. 17, no. 3. 265-320.

Sattin, A., and Rall, T.W. (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3'5'-phosphate content of guinea pig cerebral cortex slices. Molec. Pharmacol. 6, 13-23.

Schaefer, H. (1936). Untersuchugen uber den muskelaktionsstrom. Pflug. Arch. Ges. Physiol. 237, 329-355.

Schofield, J.H., Mira-Moser, F., Schorderet, M., and Orci, L. (1974). Somatostalin inhibition of rat growth hormone release in vitro in the presence of barium choride or 3-isobutyl-1-methylxanthine FEBS Lett. 46, 1721-174.

Schofield, J.G., and McPherson, M. (1974). Increase in pituitary adenosine 3'5' cyclic monophosphate content and potentiation of growth-hormone release from heifer anterior pituitary slices incubated in the presence of 3-isobutyl-1-methylxanthine. Biochem. J. 142, 295-300.

Schubert, E., Mitznegg, P., Strunz, U., Domschke, W., Domschke, S., Wuensch, E., Jaeger, E., Demling, L., and Heim, F. (1975). Influence of the hormone analog 13-norleucine-motilin and of 1-methyl-3-isobutylxanthines on tone and cyclid 3'5' AMP content of antral and duodenal muscles in the rabbit. Life Sci. 16, 263-272.

Schueler, F.W. (1960). The mechanism of action of the hemicholiniums. Intl. Rev. Neurobiol. 2, 77-97.

Schultz, J., and Hamprecht, B. (1973). Adenosine 3'5' monophosphate in cultured neuroblastoma cells. Effect of adenosine, phosphodiesterase inhibitors and benzazepines. Naunyn-Schmiedeberg's Arch. Pharmacol. 278, 215-225.

Sevcik, C., and Narahashi, T. (1972). Electrical contraction coupline in skeletal muscle treated with ethylene glycol. J. Gen. Physiol. 60, 221-236.

Sheterline P., Schofield, J.G., and Mira, F. (1975). Colchicine binding to bovine anterior pituitary slices and inhibition of growth-hormone release. Biochem. J. 148, 435-459.

Shinnick, P.L. (1974). Ph.D. dissertation. Sites of action of theophylline at the neuromuscular junction of the cat. Loyola University.

Shinnick-Gallagher, P., and Jacobs, R.S. (1976). An analysis of the sub-synaptic site of action of theophylline on isolated cat tenuissimus muscle. J. Pharmacol. Exp. Ther. 197, 647-652.

Shinnick, P.L., and Jacobs, R. (1971). The facilitatory sites of action of theophylline in isolated cat tenuissimus muscle. Pharmacologist. 13, 264.

Shinnick, P.L., and Jacobs, R.S. (1972). The facilitatory actions of xanthines on neuromuscular transmission. Fifth Intern. Cong. Pharmacol. 5, 211.

Shirachi, D.Y., Chan, S.L., and Trevor, A. (1973). Isolation and characterization of pharmacological receptors. In: Featherstone, R.M. (ed.): A Guide to Molecular Pharmacology-Toxicology, Part I, 147-199. Marcel Dekker, Inc., N.Y.

Siggins, G.R., Hoffer, G.J., and Ungerstedt, U. (1974). Electrophysiological evidence for involvement of cyclic adenosine monophosphate in dopamine responses of caudate neurons. Life Sci. 15, 779-792.

Slater, P. (1971). The estimation of the "free" and "bound" acetylcholine content of rat brain. J. Pharm. Pharmacol. 23, 514-518.

Snell, R.S., and McIntyre, N. (1956). Changes in the histochemical appearances of cholinesterase at the motor endplate following denervation. Brit. J. Exp. Path. 37, 44-48.

Standaert, T.G., Dretchen, K.L., Skirboll, L.R., and Margenroth, V.H. (1976a). Effects of cyclic nucleotides on mammalion motor nerve terminals. J. Pharmacol. Exp. Ther. 199, no. 3, 544-552.

Standaert, F.G., Dretchen, K.L. Skirboll, L.R., and Margenroth, V.H. (1976b). A role of cyclic nucleotides in neuromuscular transmission. J. Pharmacol. Exp. Ther. 199, no. 3, 553-564.

Standfield, P.R. (1970). The effect of tetraethylammonium on the delayed currents of frog skeletal muscle. J. Physiol. (London). 209, 209-229.

Statham, H.E., Duncan, C.J., and Smith, J.L. (1976). The effect of the ionophore A23187 on the ultrastructure and electrophysiological properties of frog skeletal muscle. Cell Tiss. Res. 1973, 193-209.

Steinbach, H.B., and Spiegelman, S. (1943). The sodium and potassium balance in squid nerve axoplasm. J. Cell. Comp. Physiol. 22, 187-196.

Straughan, D.W. (1960). The release of acetylcholine from mammalian motor nerve endings. Brit. J. Pharmacol. 15, 417-422.

Strauss, A.J.L., Seegal, B.C., Hsw, K.C., Burkholder, P.M., Mastuk, W.L., and Osserman, K.E. (1960). Immunoflourescence demonstration of muscle binding, complement fixing serum globulin fraction in myasthenia gravis. Proc. Soc. Exp. Biol. Med. 105, 184-191.

Sutherland, E.W., Robinson, G.A., and Butcher, R.W. (1968). Some aspects of the biological role of adenosine 3', 5'-monophosphate (cyclic AMP). Circ. 3, 279-306.

Takeuchi, N. (1963). Effects of calcium on the conductance change of the endplate membrane during the action of transmitter. J. Physiol. (London). 167, 141-155.

Takeuchi, A., and Takeuchi, N. (1959). Active-phase of frog's endplate potential. J. Neurophysiol. 22, 395-411.

Takeuchi, A., and Takeuchi, N. (1960a). Further analysis of relationships between endplate potential and endplate current. J. Neurophysiol. 23, 397-402.

Takeuchi, A., and Takeuchi, N. (1960b). On the permeability of endplate membrane during the action of transmitter. J. Physiol. (London). 154, 52-67.

Takeuchi, A., and Takeuchi, N. (1962). Electrical changes in the pre- and postsynaptic axons of the giant synapse of Loligo. J. Gen. Physiol. 45: 1181-1186.

Tasaki, I. (1939). The electro-saltatory transmission of the nerve impulse and the effect of narcosis upon nerve fiber. Am. J. Physiol. 127, 211-227.

Teng, C.T., and Teng, C.S. (1977). Studies on sex-organ development. Biochem. J. 162, 123-124.

Thesleff, S. (1955). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. Acta. Physiol. Scand. 34, 218-231.

Thesleff, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. J. Physiol. (London). 151, 598-607.

Thesleff, S., and Quastel, D.M.J. (1965). Neuromuscular pharmacology. Ann. Rev. Pharmacol. 5, 263-284.

Torda, C., and Wolff, H.G. (1945). Decomposition products of nucleoproteins and related substances and muscle sensitivity to acetylcholine and potassium. Proc. Soc. Exp. Biol. Med. 58, 29-31.

Triggle, D.J., and Triggle, C.R. (1976). Chemical Pharmacology of the Synapse. Academic Press, London, New York.

Turkanis, S.A. (1973). Some effects of vinblastine and colchicine on neuromuscular transmission. Brain Research. 54, 324-329.

Usdine, E. (1970). Reactions of Cholinesterases with substrates, Inhibitors and Reactivators. In: Anticholinesterase Agents, 1 (A.G. Karczmar. ed.) 47-354, Permagon, Oxford.

Ussing, H.H. (1947). Interpretation of the exchange of radiosodium in isolated muscle. Nature. (London). 160, 262-263.

Van Inwegan, R.G., Robison, G.A., and Thompson, W.J. (1975). Cyclic nucleotide phosphodiesterases and thyroid hormones. J. Biol. Chem. 250, no. 7, 2452-2456.

Varagic, V.M., and Zugic, M. (1971). Interactions of xanthine derivatives, catecholamines and glucose-6-phosphate on the isolated phrenic nerve diaphragm preparation of the rat. Pharmacol. 5, 275-286.

Vedeckis, W.Y., Bollenbacker, W.E., and Gilbert, L.I. (1976). Insect prothoracic glands: A role for cAMP in the stimulation of alpha-ecdysone secretion. Mol. Cell. Endocrinol. 5, 81-88.

Vere-Jones, D. (1966). Simple stochastic models for the release of quanta of transmitter from a nerve terminal. Aust. J. Stat. 8, 53-63.

Walther, H. (1961). Die Coffein-, Theobromin- und Theophyllinwirkung auf das isolierte Phrenicus-Zwerchfell-Praparat der Ratte bei unterschiedlichen extrazellularen Kalium- und Calciumionen-konzentrationen. Acta Biol. Med. Germ. 6, 267-278.

Walther, H. (1962). Zur Coffein-und Theophyllinwirkung am ermudeten Rattenzwerchfellpraparat. Arch. Int. Pharmacodyn. 138, 597-612.

Waser, P.G. (1960). The cholinergic receptor. J. Pharm. Pharmac. 12, 577-594.

Waser, P.G. (1967). Receptor localization by autoradiographic techniques. Ann. N.Y. Acad. Sci. 144, 737-753.

Webb, D.A., and Young, D.A. (1940). Electroylte content and action potential of the giant nerve fibres of loligo. J. Physiol. 98, 299-313.

Weber, A., and Herz, R. (1968). The mechanism of action of caffeine on sarcoplasmic reticulum. J. Gen. Physiol. 52, 760-772.

Westfall, T.C., Kitay, D., and Wahl, G. (1976). The effect of cyclic nucleotides on the release of ^{3}H-dopamine from rat striatal slices. J. Pharmacol. Exp. Ther. 199, 149-157.

Whittaker, V.P., and Dowdall, M.J. (1973). Constituents of cholinergic vesicles. I: La transmission cholinergique de 1' excitation. 101-117, Paris: INSERM.

Williams, C.D., Horner, A.K., and Catt, K.J. (1976). Effects of methyxanthines on gonadotropin-induced steroidogenesis and protein synthesis in isolated testis interstitial cells. Endocr. Res. Commun. 3, 343-358.

Wilson, D.F. (1973). Effects of caffeine on neuromuscular transmission in the rat. Am. J. Physiol. 225 (4), 862-865.

Wilson, D.F., and DeVillez, E.J. (1973). The role of cyclic AMP in the nerve terminal of the rat. Fed. Proc. 32, 334.

Wilson, D.F. (1974a). The effects of dibutyryl cyclic adenosine 3', 5'-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. J. Pharmacol. Exp. Ther. 188, 447-452.

Wilson, D.F. (1974b). Facilitation of transmitter release the mammalian neuromuscular junction. Am. J. Physiol. 227 (5), 1098-1102.

Zaimis, E. (ed.) (1976). Handbuch der Experimentellen Pharmakologie, Erganzungswerk; Neuromuscular Junction. 42, Springer-Verlag, Berlin-Heidelberg-New York.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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